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The Z-DNA Motif $d(TG)_{30}$ Promotes Reception of Information during Gene Conversion Events while Stimulating Homologous Recombination in Human Cells in Culture

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Tracts of the alternating dinucleotide polydeoxythymidylic-guanylic [d(TG)] · polydeoxyadenylic-cytidylic acid [d(AC)], present throughout the human genome, are capable of readily forming left-handed Z-DNA in vitro. We have analyzed the effects of the Z-DNA motif $d(TG)_{30}$ upon homologous recombination between two nonreplicating plasmid substrates cotransfected into human cells in culture. In this study, the sequence $d(TG)_{30}$ is shown to stimulate homologous recombination up to 20-fold. Enhancement is specific to the Z-DNA motif; a control DNA fragment of similar size does not alter the recombination frequency. The stimulation of recombination is observed at a distance (237 to 1,269 base pairs away from the Z-DNA motif) and involves both gene conversion and reciprocal exchange events. Maximum stimulation is observed when the sequence is present in both substrates, but it is capable of stimulating when present in only one substrate. Analysis of recombination products indicates that the Z-DNA motif increases the frequency and alters the distribution of multiple, unselected recombination events. Specifically designed crosses indicate that the substrate containing the Z-DNA motif preferentially acts as the recipient of genetic information during gene conversion events. Models describing how left-handed Z-DNA sequences might promote the initiation of homologous recombination are presented.

Homologous recombination is the process of exchange of genetic information between stretches of DNA containing sequence similarity. Although the degree of recombination is generally proportional to the length of homology, there are regions of DNA in which the rate of exchange is nonlinear with respect to distance, resulting in disparity between genetic and physical maps. The sequences responsible for this disparity, called "recombination hotspots," increase the rate of genetic exchange between homologous DNA molecules. The 8-base-pair (bp) Chi sequence in *Escherichia coli* is the most fully characterized recombination hotspot, shown to increase recombination in bacteriophage lambda and *E. coli* DNA via the RecA-RecBCD pathway (reviewed in reference 34). Recombination hotspots have also been described in fungi (12, 25) and in mammalian cells (37; W. P. Wahls, L. J. Wallace, and P. D. Moore, Cell, in press). A short fragment of mammalian DNA from the human β-globin gene cluster, MG-1 (29), promotes unusual recombination events during yeast meiosis (41). Molecular evidence indicates that the DNA sequence responsible is an 80-bp stretch of the simple repeating dinucleotide polydeoxythymidylic-guanylic · polydeoxyadenylic-cytidylic acid, abbreviated hereafter as $d(TG)_n$.

The repeating dinucleotide $d(TG)_n$ is not detectable in the genomes of eubacteria, archaebacteria, or mitochondria (11) but is ubiquitously present in eucaryotic chromosomes (13, 29, 40). It is estimated that there are on the order of 10^5 copies of this dinucleotide repeat, each approximately 10 to 50 bp in length, scattered throughout the mammalian genome. A striking feature of these reiterated sequences is that they readily adopt the left-handed Z-DNA conformation under the appropriate conditions in vitro (14, 30, 33), and there is evidence that left-handed DNA is found in vivo in transcriptionally active chromosomes (22). The existence of Z-DNA-binding proteins in *Drosophila melanogaster* and wheat germ (21) and in mammalian cells (8) supports the notion that the left-handed DNA sequences do serve some biological function. A growing body of evidence indicates that Z-DNA is involved in homologous recombination. Purified recl enzyme from *Ustilago maydis*, like recA of *E. coli*, is capable of promoting both synapsis and strand exchange between homologous DNA molecules in vitro (17). The initial synapsis between single-stranded and duplex DNA promoted by recl involves extensive unwinding of the duplex. The resulting paranemic joint contains Z-DNA (18). Subsequently it was shown that the recl protein can promote homologous pairing between two duplex DNA molecules that contain Z-DNA (19). Addition of anti-Z-DNA antibodies or competitor Z-DNA to the reaction or altering the superhelical density of the plasmids inhibited pairing, indicating that Z-DNA is required for recl-mediated pairing of duplex molecules. The high affinity of recl for Z-DNA (16) has led to attempts to purify mammalian recombination enzymes by Z-DNA affinity chromatography. Partial purification of a human enzyme with strand transferase activity has been achieved with this strategy (8), implying that Z-DNA is involved in homologous recombination in mammalian cells. Other evidence about the role of Z-DNA in recombination has come from in vivo experiments. The Z-DNA motif $d(TG)_n$ has been shown to stimulate intramolecular homologous recombination in simian virus 40 (SV40) viral DNA replicating in somatic cells (3, 38) and to promote unusual meiotic recombination events in *Saccharomyces cerevisiae* (41).

In this study we have analyzed the effects of $d(TG)_{30}$ upon

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homologous recombination between two nonreplicating plasmids introduced into human cells in culture. The ability of mammalian cells to mediate homologous recombination between exogenously introduced plasmids has been extensively used to study the mechanisms of recombination in mammalian cells (reviewed in reference 39). We have constructed a number of substrates based upon the pSV2neo shuttle vector (36) and bacteriophage M13 that enable us to analyze the effects of specific DNA sequences upon homologous recombination. We report here that the sequence $d(TG)_{30}$ enhances homologous recombination in human cells up to 20-fold and that the enhancement of gene conversion events involves preferential reception of genetic information by the $d(TG)_{30}$-containing molecule.

MATERIALS AND METHODS

Cells. The human bladder carcinoma cell line EJ was obtained from Raju Kucherlapati, and COS-1 cells were provided by Kiranur Subramanian (College of Medicine, University of Illinois at Chicago). Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Transfections. DNA was introduced into EJ cells by the calcium phosphate precipitation method of Lowy et al. (24), in the absence of carrier DNA. Cells (5×10^5) were plated on 60-mm tissue culture dishes 16 h prior to transfection. A precipitate containing the appropriate DNA(s) was placed on the cells for 4 h. The cells were then treated for 2 min with 20% dimethyl sulfoxide in 138 mM NaCl–5 mM KCl–0.7 mM Na_2HPO_4–6 mM dextrose–20 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), pH 6.92, and were washed three times with Hanks balanced salt solution. The transfected cells were incubated for 24 h in the absence of selection and then transferred to 100-mm plates for selection in Dulbecco modified Eagle medium containing G418 at 400 μg/ml. $G418^r$ colonies were visible 7 to 10 days after transfection. At 2 to 3 weeks posttransfection, the plates were stained or individual colonies were isolated and expanded for further analysis.

Cell fusions. Cell fusions between $G418^r$ transfectants and monkey COS-1 (10) cells were carried out by the method of Davidson et al. (6). Approximately 10^6 of each cell type was plated on 100-mm dishes and allowed to attach for 16 h. The medium was removed, and the cells were treated for 2 min with 50% polyethylene glycol, M_W 1,000, and washed three times with Hanks balanced salt solution; the medium was then replaced. Low-molecular-weight DNA was isolated 48 h later.

DNA manipulations. Low-molecular-weight DNA from the COS-1 cell fusions was isolated by the procedure of Hirt (15). The low-molecular-weight DNA was introduced into the *recA* mutant *E. coli* DH-5 by standard methods (26). Plasmid DNA was made and isolated by conventional procedures (27). DNA for transfections was purified by cesium chloride density gradient ultracentrifugation. Restriction endonuclease and other DNA-modifying enzymes were used according to the recommendations of the manufacturer (New England BioLabs, Inc., or Bethesda Research Laboratories, Inc.). Plasmid DNA was fractionated on 0.8% agarose gels.

RESULTS

Homologous recombination substrates. The recombination substrates used and the relevant restriction enzyme sites are shown in Fig. 1. All the substrates were derived from the parental plasmid pSV2neo, which contains the complete *neo* gene from the bacterial transposon Tn*5* under the control of the SV40 early promoter and poly(A) addition signals. This enables the plasmid to confer resistance to kanamycin or neomycin when in bacterial cells and resistance to the aminoglycoside analog G418 when in mammalian cells (36). Two types of deletion plasmids, designated DL (deletion left) and DR (deletion right), have been constructed within the *neo* gene by removing 248 bp of 5′ coding sequences and 283 bp of 3′ coding sequences, respectively (20). Neither of the deletion plasmids is capable of conferring resistance to kanamycin or G418. The plasmids DL and DR contain 501 bp of homology between the sites of the two deletions. A homologous recombination event within this interval between the DL and DR molecules can give rise to a functional wild-type *neo* gene. Restriction site polymorphisms have been introduced into the deletion plasmids, either by linker addition or by filling and religating restriction enzyme sites, to generate the equivalent of heteroallelic markers (35). This enables us to analyze the products of recombination and localize the sites of the recombination events. An 81-bp *Hin*dIII-*Hin*cII fragment from the plasmid pDHF14, containing $d(TG)_{30}$, was cloned into the *Stu*I-*Hin*dIII sites of pSV2neo and the two deletion plasmids pLCKS (DLTG) and pDRD86 (DRTG). All molecules therefore contained the Z-DNA motif in the same orientation with the $d(AC)_{30}$ in the top strand 5′ of the *neo* gene. The control plasmid DRCH contained the 210-bp *Pst*I-*Hin*dIII fragment of yeast DNA from YRp7. In addition to the plasmid substrates, we constructed M13-based recombination substrates. The entire wild-type *neo* gene coding region, excised from pSV2neo between the *Hin*dIII and *Sma*I sites, has been cloned into the replicative form of M13mp18 to generate Mneo-5. Mneo-5 lacks both the 5′ and 3′ control sequences necessary for expression in mammalian cells and cannot confer resistance to G418. The $d(TG)_{30}$-containing fragment was cloned between the *Fsp*I and *Hin*dIII sites to generate MneoTG. The resulting Mneo-5 substrates contain homology to the pSV2neo plasmid substrates only within the *neo* gene, and the location of the $d(TG)_{30}$ insert is on the periphery of that homology (Fig. 1).

$d(TG)_{30}$ does not alter transfection efficiencies. The DNA substrates were individually introduced into human EJ cells by calcium phosphate precipitation. Following a 2-week selection in medium containing G418, the plates were stained and the colonies were counted to generate the data presented in Table 1. The presence of $d(TG)_{30}$ did not alter the transfection efficiency of pSV2neo. Transfection with either of the deletion plasmids always failed to yield $G418^r$ colonies, regardless of whether or not they contained the $d(TG)_{30}$ insert. The Mneo-5 and MneoTG substrates also failed to yield colonies, despite the fact that they contain the complete coding region of the *neo* gene. This is not surprising, however, because the *neo* gene would have to integrate into the genome in such a fashion as to provide both the missing 5′ and 3′ mammalian gene expression signals. Even in other experiments where we have added the 3′ poly(A) addition sequences to Mneo-5, we find that only about one colony is generated per 100 μg of input DNA by fortuitous integration next to genomic promoters (data not shown). These data indicate that $d(TG)_{30}$ has no effect upon transfection efficiency and subsequent gene expression and that the recombination substrates are individually incapable of giving rise to $G418^r$ colonies.

$d(TG)_{30}$ promotes homologous recombination. The assay

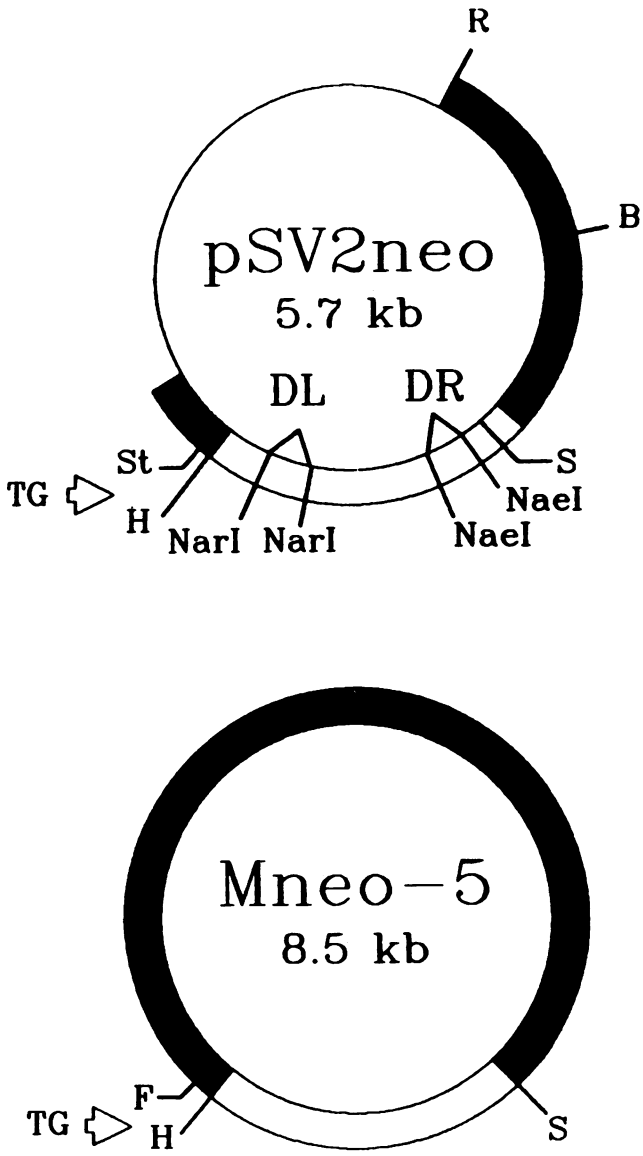

FIG. 1. DNA molecules used in this study. Circular maps of pSV2neo and Mneo-5. pSV2neo contains the *amp* gene and origin of replication from pBR322 and contains the complete *neo* gene and bacterial-expression sequences from transposon Tn*5*. The SV40 origin of replication and early promoter and SV40 poly(A) addition signals have been added, flanking the *neo* gene to allow for expression in mammalian cells. It confers resistance to the aminoglycoside analog G418 when in mammalian cells. Deletion left (DL) is created by removing the 248-bp *Nar*I-*Nar*I fragment, and deletion right (DR) is created by removing the 283-bp *Nae*I-*Nae*I fragment; both are incapable of conferring G418 resistance. Mneo-5 contains the complete coding region of the *neo* gene but lacks the promoter and 3′ processing signals necessary for mammalian gene expression. pSV2neo and Mneo-5 share homology only within the *neo* coding region. Open box, *neo* gene; TG, insertion point of Z-DNA motif d(TG)$_{30}$ or control DNA sequences; DL, position of deletion left; DR, position of deletion right; black box, SV40 control sequences including the 5′ origin of replication and promoter and the 3′ poly(A) addition signals; line, pBR322 sequences; shaded box, M13 sequences; R, *Eco*RI; St, *Stu*I; H, *Hin*dIII; S, *Sma*I; B, *Bam*HI; F, *Fsp*I. Several derivatives of the DL and DR plasmids with alterations at the restriction enzyme sites shown here were used in certain experiments to generate allelic markers (35).

TABLE 1. Effects of d(TG)$_{30}$ upon transfection efficiencies

Input DNA[a]	No. of expts	Total colonies/total μg of DNA	Colonies/μg of DNA
pSV2neo	6	753/12	62.75
pSV2neoTG	6	737/12	61.42
DL	5	0/100	<0.01
DR	5	0/100	<0.01
DRTG	5	0/100	<0.01
Mneo	5	0/100	<0.01
MneoTG	4	0/80	<0.01

[a] In each experiment 1 μg of wild-type DNA or 10 μg of substrate DNA was presented per 10^6 cells per 60-mm dish.

for homologous recombination was as previously described (20). The two deletion molecules DL and DR were mixed and introduced into the cells by calcium phosphate precipitation. In a certain proportion of the cells homologous recombination occurred between the DL and DR molecules, either a gene conversion or a reciprocal exchange event, as shown in Fig. 2, to reconstitute a wild-type *neo* gene. The plasmids then integrated randomly into the chromosomes and, after a period of time in selective medium, gave rise to G418^r colonies. The recombination frequency is calculated as the number of G418^r colonies generated per microgram of input deletion plasmid DNA divided by the number of G418^r colonies generated per microgram of wild-type pSV2neo. The presence of d(TG)$_{30}$ in the DR substrate stimulated homologous recombination 3.4-fold (Table 2). In those crosses, however, the presence of the d(TG)$_{30}$ insert in one of the recombination substrates introduced heterology between the two molecules. In order to determine whether the stimulation of recombination was specific to the d(TG)$_{30}$ insert or was a result of the heterology, we introduced a 210-bp control insert, CH, into the same location of a DR molecule and measured recombination frequencies. The presence of 210 bp of heterology in the DL × DRCH cross did not stimulate recombination, indicating that the recombination enhancement was specific to the d(TG)$_{30}$ sequence. Because the d(TG)$_{30}$ insert was located outside of the region between the two deletions, 237 bp 5′ of the nearest deletion, it is evident that d(TG)$_{30}$ can act to stimulate recombination at a distance. In order to further characterize the effects of the Z-DNA motif, we decided to introduce d(TG)$_{30}$ into the DL molecule and conduct differentially configured crosses.

Stimulation of recombination is observed with d(TG)$_{30}$ in either substrate and maximal when in both substrates. We analyzed the effects of placing the Z-DNA motif in one, the other, or both of the plasmid recombination substrates

TABLE 2. Effects of d(TG)$_{30}$ upon homologous recombination

Input DNA[a]	No. of expts	Total colonies/ total μg of DNA	Colonies/ μg of DNA	Recombination frequency (10^{-3})	Enhancement ratio[c]
pSV2neo	6	753/12	62.75		
DL × DR	14	100/750	0.133	2.12	1.0
DL × DRCH	4	26/200	0.130	2.07	1.0
DL × DRTG	11	266/590	0.451	7.19	3.4

[a] In most experiments 1 μg of wild-type DNA or 10 μg of each substrate DNA was presented per 10^6 cells per 60-mm dish.

[b] Calculated as (G418^r colonies/μg of DL + DR)/(G418^r colonies/μg of pSV2neo).

[c] Ratio of the recombination frequency of any substrate combination relative to the recombination frequency of the unsubstituted DL × DR cross.

TABLE 3. Homologous recombination frequency data from differentially configured plasmid crosses[a]

Input DNA	No. of expts	Total colonies/ total μg of DNA	Colonies/ μg of DNA	Recombination frequency (10^{-3})	Enhancement ratio
DL × DR	4	25/200	0.125	1.99	1.0
DLTG × DR	4	171/200	0.855	13.63	6.8
DL × DRTG	4	61/190	0.321	5.12	2.6
DLTG × DRTG	4	495/200	2.475	39.44	19.8

[a] The data were derived as for Table 2. Each experiment included all four substrate combinations.

introduced into the cells. The data are presented in Table 3. The maximum stimulation of recombination, 19.8-fold, was observed when the $d(TG)_{30}$ insert was present, homozygous, in both substrates. Although recombination enhancement was also observed when the insert was present in only one substrate, the degree of stimulation depended upon which substrate contained the insert. *cis* linkage of $d(TG)_{30}$ to the distal deletion stimulated recombination 2.6-fold, while *cis* linkage of the insert to the proximal deletion stimulated recombination 6.8-fold. We would not expect to observe this locational bias if the $d(TG)_{30}$ sequence was solely affecting reciprocal exchange events. Reciprocal exchanges have to occur within the same region, between the sites of the 5′ and 3′ deletions, to generate a wild-type *neo* gene, regardless of which molecule contains the insert. Gene conversion events, on the other hand, involve correction of deletions located 237 to 485 bp (DL) or 986 to 1,269 bp (DR) distant from the inserted $d(TG)_{30}$ sequence. The locational bias observed in the heterozygous crosses implied that the $d(TG)_{30}$ insert may be preferentially affecting gene conversion events. Furthermore, assuming that the effects of the sequence decreased over distance, the greater stimulation when linked to DL suggested that the Z-DNA motif was stimulating the reception of information during gene conversion events. In order to test this hypothesis, we constructed recombination substrates to specifically analyze the effects of $d(TG)_{30}$ upon gene conversion events.

$d(TG)_{30}$ promotes reception of information during gene conversion events. The strategy for assaying gene conversion is outlined in Fig. 2. The entire wild-type *neo* gene has been cloned into M13mp18 RF to generate Mneo-5, which is incapable of generating G418 resistance in mammalian cells

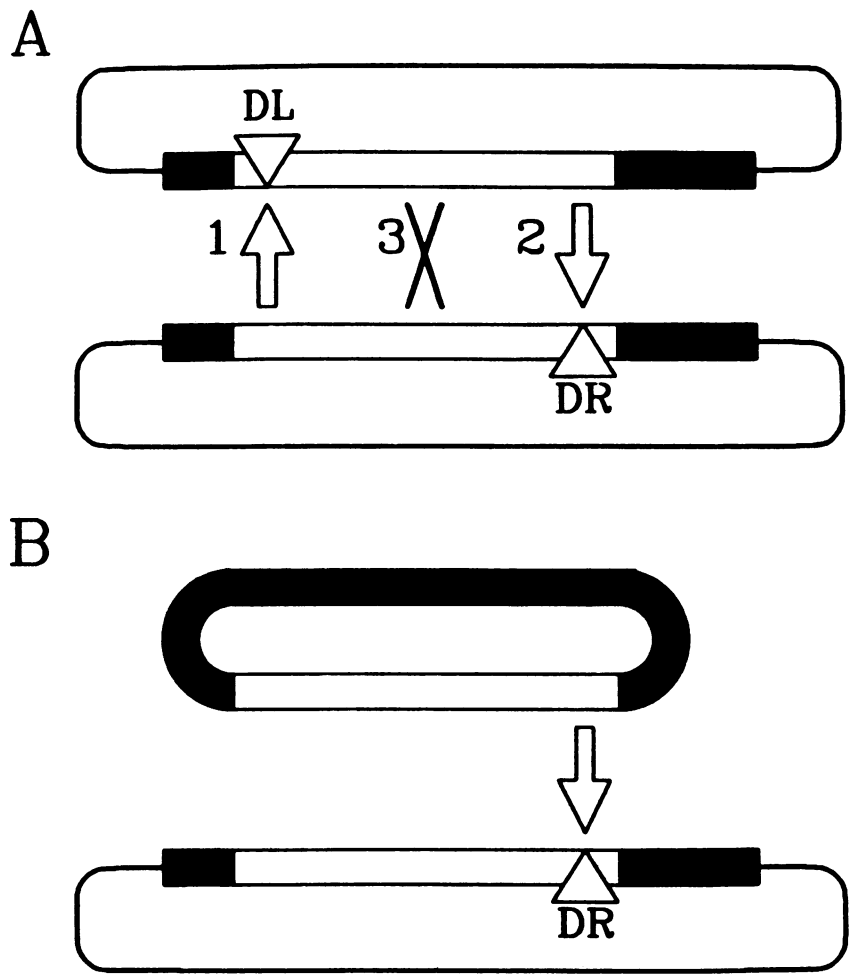

FIG. 2. Mechanisms of homologous recombination. (A) In the plasmid cross DL × DR, a wild-type *neo* gene may be reconstituted by three different homologous recombination events: (i) gene conversion of DL, (ii) gene conversion of DR, and (iii) reciprocal exchange. (B) When Mneo-5 is crossed to DR, only gene conversion of the deletion in the DR substrate molecule can give rise to a functional wild-type *neo* gene, as explained in text. Arrows, Gene conversion; X, reciprocal exchange; other symbols are described in the legend to Fig. 1.

TABLE 4. Effects of d(TG)$_{30}$ upon gene conversion in differentially configured Mneo-5 × DR crosses[a]

Input DNA	No. of expts	Total colonies/ total μg of DNA	Colonies/ μg of DNA	Recombination frequency (10^{-3})	Enhancement ratio
Mneo × DR	4	11/200	0.055	0.88	1.0 (0.44)[b]
MneoTG × DR	4	26/200	0.130	2.07	2.4
Mneo × DRTG	4	56/200	0.280	4.46	5.1
MneoTG × DRTG	4	50/200	0.250	3.98	4.5

[a] The data were derived as for Table 2.
[b] The value in parentheses shows recombination frequency relative to the frequency determined between intact plasmids in the unsubstituted DL × DR cross in Table 3.

because it lacks both the 5′ and 3′ mammalian control signals. When Mneo-5 and DR are introduced into the cells, the predominant way that a functional wild-type *neo* gene is generated is via a gene conversion event, with the Mneo-5 molecule acting as the donor of genetic information and the DR molecule acting as recipient. Two other possible modes of generating a functional *neo* gene, a double reciprocal exchange or a single reciprocal exchange with serendipitous acquisition of the missing 5′ or 3′ control elements during integration into the genome, are both low-frequency events and make negligible contributions to overall recombination frequencies. Because there is a strict directionality of information transfer required to generate a functional *neo* gene, it is possible to ascertain whether the effects of any inserted sequences are upon donation or reception of information during gene conversion events.

We introduced d(TG)$_{30}$ into the Mneo-5 substrate and analyzed its effects upon recombination between Mneo-5 and DR in differentially configured crosses (Table 4). For the unsubstituted Mneo-5 × DR cross, the recombination frequency was slightly less than half that observed in the unsubstituted DL × DR plasmid cross. Placing d(TG)$_{30}$ in the donor molecule (Mneo-5) stimulated recombination 2.4-fold, while placing d(TG)$_{30}$ in the recipient molecule (DR) resulted in a 5.1-fold stimulation. When the Z-DNA motif was present in both substrates, we observed a 4.5-fold enhancement of recombination. These data indicate that d(TG)$_{30}$ does stimulate gene conversion and preferentially stimulates when in the substrate receiving genetic information during those events.

Product analysis. In order to rescue the recombination products, we fused cells derived from individual clonally expanded G418^r colonies to COS-1 cells. Such fusions permit the excision and amplification of integrated plasmids containing an SV40 origin of replication (10). A single *neo*$^+$ colony was isolated from each clonal fusion. The resulting *neo*$^+$ plasmids were amplified in *recA* mutant *E. coli* and subject to restriction endonuclease mapping to localize the sites of recombination events. A comparison of the restriction sites in such rescued plasmids to the restriction sites in the parental molecules may be used to make predictions about the mechanisms of recombination. Figure 3 shows an example of this type of analysis. All of the restriction sites in the product molecule pTP6-3 are derived from the DL substrate, consistent with the recombination event being a gene conversion in which the deletion in the DL molecule was corrected. The map of product pTP6-4 is consistent with a gene conversion event in which the DR molecule acted as recipient, accompanied by coconversion of the *Xba*I site 58 bp away, while pTP6-24 could have been generated by either

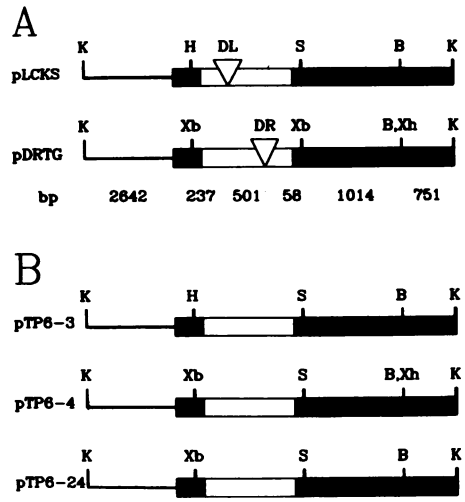

FIG. 3. Nature of recombination events based upon analysis of rescued products. (A) Linear restriction maps of recombination substrates pLCKS and pDRTG. The interval length in base pairs is shown below the maps. (Not to scale.) (B) Restriction maps of plasmids rescued from three independent G418^r colonies by COS-1 cell fusion as explained in text. On the basis of restriction site polymorphisms, the mechanism of the initial homologous recombination event may be predicted: pTP6-3, gene conversion with the pLCKS substrate acting as the recipient of genetic information; pTP6-4, gene conversion of the pDRTG substrate accompanied by coconversion of the 3′ *Xba*I site; pTP6-24, reciprocal exchange or gene conversion of pLCKS with coconversion of the *Hin*dIII site located 237 bp 5′ of the deletion. As demonstrated in Fig. 4 and 5, this type of analysis is flawed. K, *Kpn*I; Xb, *Xba*I; Xh, *Xho*I; other symbols are as described in the legend to Fig. 1.

a reciprocal exchange event or gene conversion of the DL deletion with coconversion of the *Hin*dIII site 237 bp 5′.

However, with the COS-1 rescue technique, it cannot be assumed that the recovered molecules accurately represent the original recombination products. The multiple interplasmid recombination events that occur when DNA is introduced into mammalian cells lead to integration of head-to-tail tandem arrays at various locations in the genome (32). If parental deletion plasmids are integrated in tandem arrays with the recombined *neo*$^+$ plasmid, then the excision event need not exclusively recover DNA from the product molecule. A rescued wild-type *neo* gene could be accompanied by portions of the adjacent parental molecules, which would give rise to a plasmid with a different restriction map than that of the original recombination product.

In order to test the fidelity of the excision event, we isolated eight plasmids from each of three different G418^r cell lines and determined the nature of their restriction sites. The duplicate isolates from two of the G418^r cell lines all contained identical restriction site configurations (Fig. 4). The duplicate isolates from the TP6-3 cell line, however, were heterogeneous. One of the plasmids contained a deletion, consistent with previous observations that SV40-derived replicating plasmids occasionally randomly delete nonessential regions (4, 23). Of the remaining seven plasmids, five contained restriction sites exclusively derived from the DL parental molecule, while two contained the 5′ *Xba*I site that must have come from the DR parental substrate. A model for their generation is presented in Fig. 5. We assume that the *neo*$^+$ product molecule arose via a gene conversion event of the DL molecule and is subsequently incorporated into the chromosome in a tandem array with a DR substrate molecule

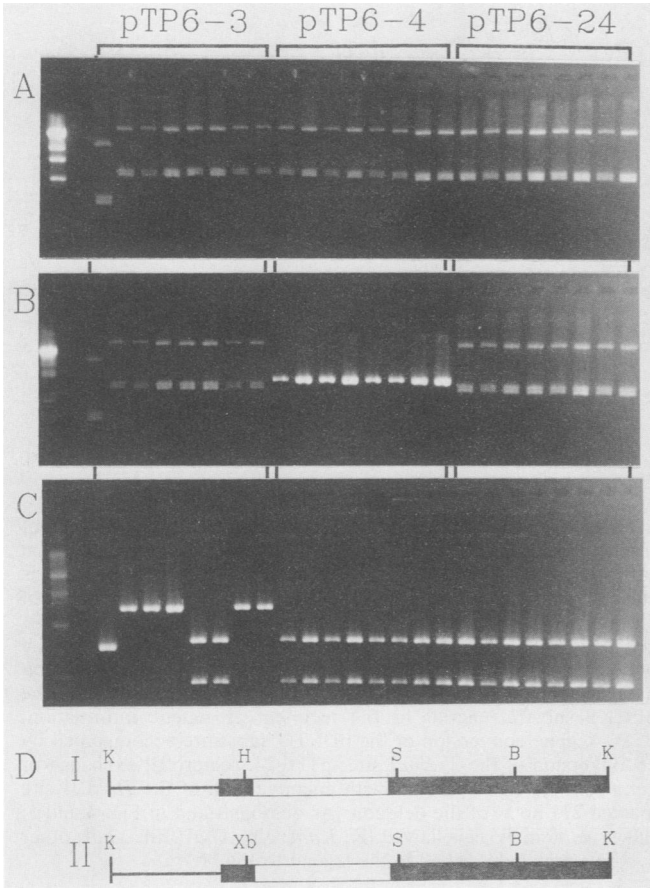

FIG. 4. Fidelity of rescue of recombination products by COS cell fusion. Clonally isolated cells from three independent G418^r colonies were expanded and fused to COS-1 cells to rescue the integrated plasmids. Low-molecular-weight DNA were made and introduced into *recA* mutant *E. coli* DH-5, which was plated on kanamycin plates to select for the wild-type *neo* gene generated by homologous recombination in the human EJ cells. Eight bacterial colonies from each were expanded, and the plasmid DNA was subjected to restriction mapping to determine whether multiple plasmid isolates from individual G418^r cells contain homogeneous restriction site patterns. Lane 1, Lambda *Hin*dIII marker; lanes 3 through 10, isolates from cell line TP6-3; lanes 11 through 18, isolates from cell line TP6-4; lanes 19 through 26, isolates from cell line TP6-24. (A) Undigested DNA. (B) *Xho*I digestion. (C) *Bam*HI and *Xba*I double digestion. (D) Restriction maps of plasmids rescued from cell line TP6-3. Type I contains restriction sites exclusively from the pLCKS parental substrate, while type II contains the 5′ *Xba*I site derived from the pDRTG parental substrate. Thus the rescued plasmids, selected for the wild-type *neo* gene generated by homologous recombination, cannot be used to make predictions about the nature of the initial recombination event because they may contain restriction sites that were not present in the initial recombination product. Restriction sites are as defined for Fig. 6.

to its right. Two different excision events, each spanning the corrected *neo* gene, give rise to plasmids with two different restriction site configurations. Although only two excision events are depicted, this type of excision event could occur between any of the restriction site polymorphisms, resulting in rescued molecules that do not accurately indicate the mechanism of the original recombination event.

Because there were at least two separate homologous recombination events, one to generate the wild-type *neo* gene prior to integration in the chromosome, and the second upon excision from the chromosome, we were unable to classify the recombination events as gene conversion or reciprocal exchange, based upon the restriction maps of the rescued products. It is, however, possible to determine the overall frequency of recombination events within each interval. Those data are presented in Fig. 6. Because selection

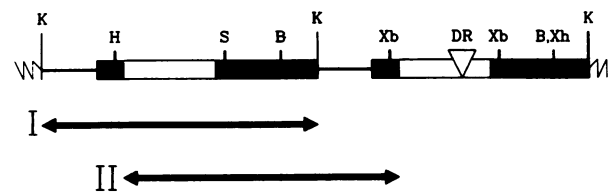

FIG. 5. Model for inaccurate excision of integrated recombination products. The *neo*$^+$ homologous recombination product in cell line TP6-3 is assumed to have arisen by a gene conversion event that corrected the deletion in the pLCKS parental substrate molecule. Because plasmids introduced into mammalian cells become integrated into the chromosome in tandem arrays (32), the recombination product may become incorporated with a *neo* mutant parental pDRTG substrate molecule to its right. Excision events during COS-1 cell fusion rescue, depicted by arrows I and II, can generate plasmid molecules that contain the *neo*$^+$ gene but are heterogeneous with respect to other markers. Although only a dimeric integrant and two excision events are depicted, this type of event could lead to alteration of any of the restriction sites in the actual recombination product. Restriction sites are as defined for Fig. 6.

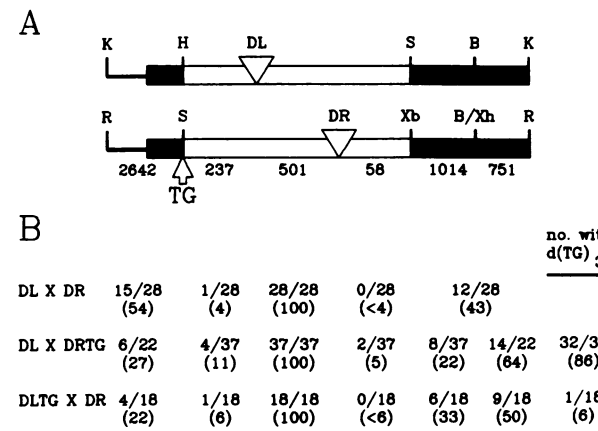

FIG. 6. Position and frequency of recombination events in rescued plasmids. (A) Linear restriction map of recombination substrates pLCKS (DL) and pLCX102 (DR): TG, position of Z-DNA motif d(TG)$_{30}$ insertion; K, *Kpn*I; R, *Eco*RI; H, *Hin*dIII; S, *Sma*I; DL, deletion left; DR, deletion right; Xb, *Xba*I; B, *Bam*HI; Xh, *Xho*I. The interval size in base pairs is shown below the map (not to scale). In some crosses, substrates with different restriction site markers were used. (B) Frequency of recombination events within each interval and inheritance of d(TG)$_{30}$ in rescued products. The data were derived from restriction enzyme mapping and agarose gel electrophoresis of recombination products rescued from G418^r cells as described in the text. Each plasmid was scored as recombinant for a given interval if the restriction enzyme sites bordering that interval were derived from the two different parental substrate molecules. The number of plasmids containing recombination events in each interval is indicated, and the percentage is shown in parentheses. Frequencies in intervals other than between DL and DR (the selected interval) indicate secondary unselected recombination events. The inheritance of the Z-DNA motif within the products is shown in the right-hand column.

was for restoration of a wild-type *neo* gene, the interval between the deletions showed 100% recombination in every cross. Homologous recombination events also occurred within the other intervals at a relatively high frequency, either in addition to the selected event during the initial round of recombination or during the excision event as described previously. The presence of d(TG)$_{30}$ altered the distribution and frequency of these secondary unselected recombination events. In crosses containing the Z-DNA motif, secondary recombination events were decreased in the *Eco*RI-*Hin*dIII interval and increased in the interval between the d(TG)$_{30}$ insert and the selected interval. In addition, the products of one cross, DL × DRTG, showed a 5% recombination frequency within the short 58-bp interval between the right deletion and the 3′ *Sma*I site.

Also shown in Fig. 6 is the inheritance of the d(TG)$_{30}$ sequence in the rescued products. The majority isolated from the DL × DRTG cross (86%) contained the d(TG)$_{30}$ sequence, while only 6% of the DLTG × DR products contained the Z-DNA motif. This distribution, however, is not unusual for our system. If the recombination event were a single reciprocal exchange, we would expect to recover the d(TG)$_{30}$ sequence in the *neo*$^+$ product only if it was originally present in the DR substrate. Furthermore, gene conversion events of the DL deletion frequently involve coconversion of the 5′ *Hin*dIII locus, while coconversion of the 3′ *Xba*I locus frequently occurs when the DR deletion is converted. In the DLTG × DR cross, for example, if the DR molecule is corrected, the product will not contain d(TG)$_{30}$. If the DL deletion is converted to the wild type, we would expect high-frequency coconversion of the d(TG)$_{30}$ locus to the wild type as well. Within the limits imposed by sample size, the observed distribution of d(TG)$_{30}$ in the products does not deviate from the expected distribution. Thus, while the Z-DNA motif appears to increase the frequency of multiple recombination events, it does not appear to alter the proportion of coconversion events.

DISCUSSION

In this study it is demonstrated that the Z-DNA motif d(TG)$_{30}$ stimulates homologous recombination between two nonreplicating plasmid substrates in human cells in culture. Stimulation is observed when d(TG)$_{30}$ is present in either of the substrates and reaches a maximum stimulation of 20-fold when it is present in both substrates. Two observations indicate that the effect is a result of the d(TG)$_{30}$ insert and not heterology per se: first, the presence of random heterology in the cross DL × DRCH does not alter the recombination frequency; and second, the cross DLTG × DRTG, containing no heterology, yields maximum recombination enhancement. Because the d(TG)$_{30}$ insert was located outside of the region within which recombination must occur to generate a wild-type *neo* gene, 237 to 1,269 bp 5′ of the endpoints of the two deletions, it is clear that the stimulation conferred acts at a distance. We have not analyzed the effects of the d(TG)$_{30}$ sequence in the opposite orientation.

Cloning the sequences between the *Stu*I and *Hin*dIII sites places them within the 5′ untranslated region of the *neo* gene and could conceivably alter gene expression or random nonhomologous incorporation of the plasmids into the genome, but this appears not to be the case. In this study, wild-type pSV2neo plasmids containing an insert generated colonies at the same frequency as unsubstituted plasmids; therefore, any effects upon gene expression and random integration would have to be strictly compensatory. Because it has been demonstrated that a 150-bp insert in the same region of an SV40 virus mutant does not affect viral replication or gene expression (7), it is highly unlikely that our inserts are altering both random integration and gene expression in a compensatory manner. In any event, the frequency of colony generation remains an accurate indication of the relative homologous recombination frequency.

The recombination frequencies from the heterozygous plasmid crosses indicate that there is a locational bias involved in d(TG)$_{30}$-stimulated recombination. When d(TG)$_{30}$ was present in the DL molecule, the stimulation was greater than when it was present in the DR molecule. Because reciprocal exchanges have to occur within the same interval regardless of which substrate contains the insert, these data implied that an effect of d(TG)$_{30}$ may be to stimulate gene conversion events and that there may be a preferred direction of information transfer involved. To address this issue, we configured crosses that could generate a wild-type *neo* gene only via a unidirectional gene conversion event. The recombination frequency between Mneo-5, the donor of genetic information, and DR, the recipient, was 44% of that observed between the unsubstituted plasmid molecules. These data imply that gene conversion events account for approximately 90% of the homologous recombination events between nonreplicating substrates in mammalian cells, consistent with previous observations (9) and confirmed by analysis of product configurations within genomes of G418^r colonies by polymerase chain reaction (Wahls et al., in press). Presence of d(TG)$_{30}$ in the donor molecule stimulated recombination 2.4-fold, while placing the insert in the recipient molecule stimulated it 5.1-fold, indicating that d(TG)$_{30}$ does stimulate gene conversion events and that the mechanism involves preferential reception of genetic information during those events. Because the Z-DNA motif promoted recombination 20-fold in the plasmid crosses (reciprocal exchange plus gene conversion of DL plus gene conversion of DR) and only 5-fold in the unidirectional gene conversion crosses (gene conversion of DR), it appears that d(TG)$_{30}$ also promotes reciprocal exchange events. Analysis of recombination products within the chromosomes of G418^r cells by polymerase chain reaction indicates that the d(TG)$_{30}$ sequence stimulates both gene conversion and reciprocal exchange events to an equal extent in the plasmid × plasmid crosses (W. P. Wahls and P. D. Moore, unpublished observations).

It was previously demonstrated that presence of d(TG) tracts in both homologs of chromosome XV of the yeast *S. cerevisiae* stimulated meiotic recombination sevenfold (41). Intriguingly, a large proportion of the recombinants from the d(TG) crosses required multiple recombination events in the vicinity of the Z-DNA motif. In this study we also observed multiple recombination events promoted by the d(TG)$_{30}$ sequence, although it is not possible to determine whether they occurred during the selected recombination event or independently. Due to the fact that recombination in the longer intervals approached 50%, it was not possible to determine whether any gradient of stimulation was present relative to the location of the Z-DNA motif. However, whereas in yeast meiosis there appeared to be no directional bias to gene conversion events, we observe that during d(TG)$_{30}$-promoted recombination in human somatic cells, the substrate bearing d(TG)$_{30}$ preferentially acts as the recipient of genetic information. Although the reason for this difference is unclear, our observations of directional bias during gene conversion events in somatic cells contribute to

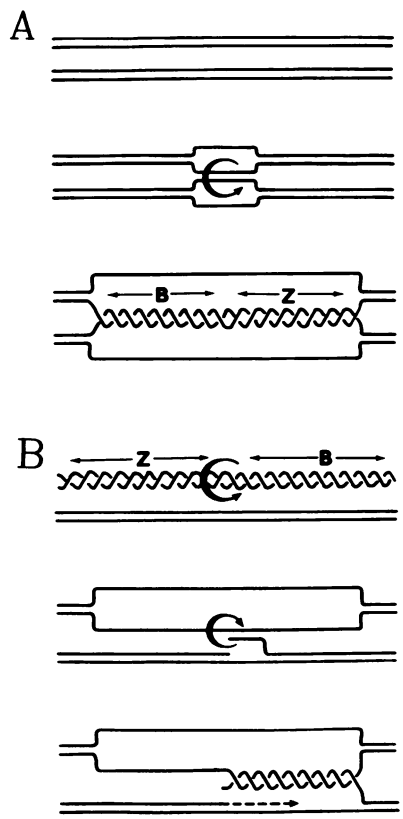

FIG. 7. Models of how Z-DNA may promote homologous recombination. (A) Formation of Z-DNA may increase the extent of paranemic joint formation during synapsis between two molecules by compensatory rotation of the participating strands (reviewed in reference 1). This would increase the length of heteroduplex DNA formed without altering the topological constraints or requiring free rotation of the participating molecules. For the sake of simplicity, only the interaction of two strands is shown; however, the other strands may also participate in paranemic joint formation. (B) Unwound duplex at the junction between left-handed and right-handed regions of the helix may facilitate interaction with free single-strand ends. Since the formation of heteroduplex initiates adjacent to the Z-DNA region, resolution of the resulting structure by the recombination model of Meselson and Radding (28) can account for preferential reception of information by the Z-DNA-containing molecule during gene conversion events.

models of how the sequence operates as a recombination hotspot.

Two mechanisms by which Z-DNA may promote homologous recombination are consistent with the results of this study. Homologous recombination involves the formation of heteroduplex DNA. In the absence of breaks or points of free rotation in the substrate molecules, only limited regions of paranemically wound heteroduplex can be formed between two duplexes. The most commonly accepted mechanism for the involvement of Z-DNA in promoting recombination is that formation of a left-handed helix adjacent to a right-handed helix can increase the extent of heteroduplex formation by compensatory rotation of the two participating strands (Fig. 7A) (reviewed in reference 1). Such a mechanism would require potential left-handed sequences to be present in both strands forming the heteroduplex, and the greater stimulation that we observe when $d(TG)_{30}$ is present in both plasmid substrates supports such a mechanism. Nevertheless, we also observe significant effects when $d(TG)_{30}$ is present in only one substrate. We therefore propose that the presence of left-handed sequences adjacent to a right-handed helix may allow for the opening up of a region of duplex DNA into single-stranded regions. This opening of the helix could be promoted by the action of strand exchange enzymes that preferentially bind to left-handed DNA. These regions could then participate in heteroduplex formation with a free single strand, even if the homology does not extend into the potential left-handed sequence of the original duplex (Fig. 7B). Thus, the resulting structure resembles an early intermediate in the Meselson-Radding model of recombination (28) and is ultimately resolved with the Z-DNA-containing substrate acting as the recipient of information during gene conversion events.

Alternatively, single-stranded regions generated at the boundary between B-DNA and Z-DNA helices may be acting as sites for nucleolytic action. Such a scenario would fit the double-strand break model, which proposes that the cleaved substrate acts as the recipient of information (31); however, such a mechanism seems unlikely. Double-strand breaks promote recombination within mammalian cells only if the site of the break is within or close to the region of homology in which the recombination must occur (2, 35). Linearizing unsubstituted molecules at the location where the Z-DNA motif was introduced, 237 bp away from the nearest deletion, results in little or no stimulation of recombination. Thus, if the stimulation of recombination is the result of nucleolytic action at the $d(TG)_{30}$ sequences, it would require supplemental factors in addition to such breaks.

Partial purification of a human enzyme with strand transferase activity from the extracts of T lymphoblasts has been achieved by Z-DNA affinity chromatography (8). The ability to isolate such a protein strongly implies that Z-DNA is involved in genetic recombination in mammalian cells. Recently, it has been demonstrated that recA protein can readily gain access to double-stranded DNA from a gapped region and the resulting nucleoprotein filament can promote homologous pairing and the formation of nascent synaptic intermediates between two duplex DNA molecules (5). This predicts another possible role for Z-DNA in homologous recombination—perhaps regions of Z-DNA act as sites for the nucleation of recombination enzymes. Although such an interaction remains speculative at the present time, the use of defined systems such as presented here, coupled with biochemical analyses of recombinase protein interactions with Z-DNA sequences in vitro, will be of utility in elucidating the roles of non-B-DNA structures in homologous recombination.

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