

## Homologous Recombination Enhancement Conferred by the Z-DNA Motif $d(TG)_{30}$ Is Abrogated by Simian Virus 40 T Antigen Binding to Adjacent DNA Sequences

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The Z-DNA motif polydeoxythymidylic-guanylic [d(TG)] · polydeoxyadenylic-cytidylic acid [d(AC)], present throughout eucaryotic genomes, is capable of readily forming left-handed Z-DNA in vitro and has been shown to promote homologous recombination. The effects of simian virus 40 T-antigen-dependent substrate replication upon the stimulation of recombination conferred by the Z-DNA motif  $d(TG)_{30}$  were analyzed. Presence of  $d(TG)_{30}$  adjacent to a T-antigen-binding site I can stimulate homologous recombination between nonreplicating plasmids, providing that T antigen is absent, in both simian CV-1 cells and human EJ cells (W. P. Wahls, L. J. Wallace, and P. D. Moore, *Mol. Cell. Biol.* 10:785-793). It has also been shown elsewhere that the presence of  $d(TG)_n$  not adjacent to the T-antigen-binding site can stimulate homologous recombination in simian virus 40 molecules replicating in the presence of T antigen (P. Bullock, J. Miller, and M. Botchan, *Mol. Cell. Biol.* 6:3948-3953, 1986). However, it is demonstrated here that  $d(TG)_{30}$  nine base pairs distant from a T-antigen-binding site bound with T antigen does not stimulate recombination between either replicating or nonreplicating substrates in somatic cells. The bound T antigen either prevents the  $d(TG)_{30}$  sequence from acquiring a recombinogenic configuration (such as left-handed Z-DNA), or it prevents the interaction of recombinase proteins with the sequence by steric hindrance.

The repeating dinucleotide polydeoxythymidylic-guanylic · polydeoxyadenylic-cytidylic acid, abbreviated hereafter as  $d(TG)_n$ , is found in the genomes of all eucaryotes (14, 31, 45) but is absent from the genomes of eubacteria, archaeobacteria, and mitochondria (13). It is estimated that there are approximately  $10^5$  copies of this simple repeat, each from 10 to 50 base pairs (bp) in length, in the mammalian genome. In addition to the property of forming left-handed Z-DNA under the appropriate conditions in vitro (15, 32, 37), there is evidence that they do so in vivo as well (25). Although the exact function of these sequences is unknown, it has been demonstrated that they stimulate intramolecular homologous recombination between replicating simian virus 40 (SV40) genomes (2, 42) and promote unusual recombination events during yeast meiosis (48). In addition, Z-DNA is involved in the pairing of homologous DNA molecules promoted by the *rec1* enzyme of the lower eucaryote *Ustilago maydis* (19-21), and Z-DNA affinity chromatography has been used to partially purify a human enzyme which catalyzes homologous DNA strand transfer in vitro (10). Although these observations do not rule out other possible functions of Z-DNA, they suggest that one role of  $d(TG)_n$  and other alternating purine-pyrimidine tracts is to interact with eucaryotic recombination enzymes during homologous recombination events in vivo. In the accompanying study, we demonstrated that the Z-DNA motif  $d(TG)_{30}$  promotes homologous recombination between two nonreplicating plasmid substrates in human cells in culture (49). The maximum stimulation of recombination, 20-fold, occurred when  $d(TG)_{30}$  was present in both substrates, but enhance-

ment was also observed when only one or the other of the substrates contained  $d(TG)_{30}$ . Recombination enhancement was conferred over distance, and it was demonstrated that the molecule containing  $d(TG)_{30}$  preferentially acts as the recipient of genetic information during gene conversion events.

There are currently two main obstacles to the study of homologous recombination in mammalian cells. First, it has not yet been possible to purify mammalian recombination enzymes to homogeneity and have them maintain their biological activity (10, 11, 17, 23, 27). This has been a barrier to the use of defined in vitro studies to assay the interaction of sequences such as  $d(TG)_n$  with specific recombination enzymes. Second, it is difficult to study the products of recombination events in mammalian cells because they become incorporated with parental substrate molecules into tandem arrays in the genome (34). Presence of adjacent substrate molecules can complicate Southern blotting analysis and can alter the restriction maps of products rescued from the genome (49; reviewed in reference 43). An alternative approach to the problem of product analysis is to use replicating recombination substrate molecules in a host cell line that is permissive for replication. Large numbers of extrachromosomal product molecules may be isolated, circumventing some of the difficulties associated with product analysis from nonreplicating systems. Previous studies indicate that recombination between replicating substrates occurs by the same types of mechanisms as in nonreplicating systems (1, 33, 35). In addition, it has been shown that double-strand breaks, which stimulate recombination in a variety of systems (reviewed in reference 43), are recombinogenic within replicating systems as well (1). Furthermore, the Z-DNA motif  $d(TG)_n$  has been shown to be recombinogenic when present in replicating SV40 genomes in COS-1 cells, although the mechanism has not been elucidated (2,

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42). These features, coupled with a relatively short time frame (with respect to nonreplicating systems), make the analysis of homologous recombination between replicating plasmids attractive for certain experiments.

In this study we assayed the ability of  $d(TG)_{30}$  to promote homologous recombination between two replicating plasmids in COS-1 cells in culture, with the original intention of analyzing the products to determine the nature of  $d(TG)_{30}$ -stimulated recombination in mammalian cells. Remarkably, we did not detect any stimulation of homologous recombination caused by presence of the  $d(TG)_{30}$  sequence. We propose that binding of T antigen (T-ag) to the SV40 origin of replication, immediately adjacent to the site of  $d(TG)_{30}$  insertion, hinders interaction of a recombinase with the  $d(TG)_{30}$  sequence, either by steric hindrance or by stabilizing B-DNA and inhibiting Z-DNA formation.

## MATERIALS AND METHODS

**Cells.** Simian CV-1 cells, COS-1 cells, and human CCD156 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum in a 7%  $CO_2$  atmosphere.

**DNA manipulations.** 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.) or as previously described (30). All DNAs used for transfection were made as previously described (30) and were purified by cesium chloride density gradient ultracentrifugation followed by extensive dialysis against 10 mM Tris hydrochloride-1 mM EDTA, pH 7.4. Low- $M_w$  DNA was introduced into the *recA* mutant *Escherichia coli* DH-5 by standard methods (29).

**Recombination substrates.** The recombination substrates are derived from the procaryotic-eucaryotic shuttle vector pSV2neo (40), which contains the neomycin phosphotransferase gene from transposon Tn5 flanked by the SV40 early promoter and poly(A) addition signals. The *neo* gene confers resistance to kanamycin and neomycin in bacteria and resistance to the aminoglycoside analog G418 when in mammalian cells. Two deletion plasmids, designated DL (deletion left) and DR (deletion right), have been constructed by removing the 248-bp *NarI-NarI* fragment and the 283-bp *NaeI-NaeI* fragment, respectively (22). There are 501 bp of homology between the sites of the two deletions; recombination events in this interval between DL and DR plasmids can regenerate a wild-type *neo* gene that can confer G418 resistance in mammalian cells. Derivatives of DL and DR containing multiple restriction site modifications are available (38). Sequences under investigation were cloned between the *StuI* and *HindIII* sites of pSV2neo and the two deletion plasmids pLCKS (DLTG) and pDR-D86 (DRTG). This places the insert outside of the region of homology within which the recombination event must occur, 237 bp from the site of the nearest deletion.

**Transfections.** Transfection of COS-1 cells was by the DEAE-dextran method (26, 44). Cells ( $10^6$ ) were placed on 100-mm tissue culture plates 18 h prior to transfection. The medium was removed, and 1 ml of serum-free Dulbecco modified Eagle medium containing 400  $\mu$ g of DEAE-dextran per ml ( $M_w$ , 500,000) and DNA was layered on the cells and incubated with occasional agitation for 3 h. The medium was removed, and the cells were treated for 2 min with 10% dimethyl sulfoxide in 138 mM NaCl-5 mM KCl-0.7 mM  $Na_2HPO_4$ -6 mM dextrose-20 mM HEPES (*N*-2-hydroxyeth-

ylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.92) (dimethyl sulfoxide) and washed three times with Hanks balanced salt solution. The cells were fed Dulbecco modified Eagle medium containing serum and were incubated for 48 h. Low- $M_w$  episomal DNA was then isolated by the procedure of Hirt (16).

DNA was introduced into CV-1 and CCD156 cells by the calcium phosphate precipitation method of Lowy et al. (28), in the absence of carrier DNA. Cells ( $5 \times 10^5$ ) 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 10% dimethyl sulfoxide and 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  $\mu$ g/ml. G418<sup>r</sup> colonies were visible 7 to 10 days after transfection. At 2 to 3 weeks posttransfection, colonies were fixed with 3.7% formaldehyde and stained with Giemsa stain.

**Replication assay.** The replication assay was essentially as previously described (3, 24, 47, 52). A 100-ng portion of experimental plasmid and 50 ng of internal replication control plasmid p2X21ori were introduced into  $2 \times 10^6$  COS-1 cells per 100-mm tissue culture dish by the DEAE-dextran transfection procedure detailed above. After the dimethyl sulfoxide shock, the cells were rinsed five times with 5 ml of Hanks balanced salt solution to remove DNA that had not entered the cells. The medium was replaced, and the plates were incubated for 56 h. Low- $M_w$  DNA was isolated by a slightly modified procedure previously described (16). One-fourth of the resulting DNA from each sample was incubated for 16 h with 5 U of *SaII*, which cuts only once within each plasmid, and 15 U of *DpnI*, which cleaves nonreplicated plasmids into multiple fragments. The digested DNA was fractionated on 0.7% agarose gels for 7 h at 60 V and transferred to a GeneScreenPlus membrane (Dupont, NEN Research Products) for 24 h in  $10 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as previously described (39). Hybridization was carried out at 65°C in  $6 \times$  SSC-1% sodium dodecyl sulfate containing  $5 \times 10^5$  cpm of pBR322 probe, prepared by the random hexanucleotide primer method, for 16 h. The membrane was washed extensively in  $2 \times$  SSC-1% sodium dodecyl sulfate at 65°C, and this was followed by two 15-min washes in 0.1% SSC at room temperature; it was then dried at room temperature. XAR-5 film was exposed for 2 to 5 h at -80°C with an intensifying screen. The bands on the resulting autoradiograph were quantified by densitometry on an LKB Ultrascan XL laser densitometer. Replication efficiency was gauged by the relative intensity of the experimental plasmid band compared with the total intensity of experimental plasmid and internal control bands.

## RESULTS

**Experimental strategy.** The strategy for analyzing homologous recombination between replicating plasmids in mammalian cells relies upon three components: derivatives of the procaryotic-eucaryotic shuttle vector pSV2neo (40); the simian cell line COS-1, which constitutively produces SV40 large T-ag and supports the replication of plasmids bearing the SV40 origin of replication (12); and the *recA* mutant *E. coli* DH-5, which is defective in its major pathway for recombination.

The two pSV2neo deletion plasmids pLCKS (DL) and

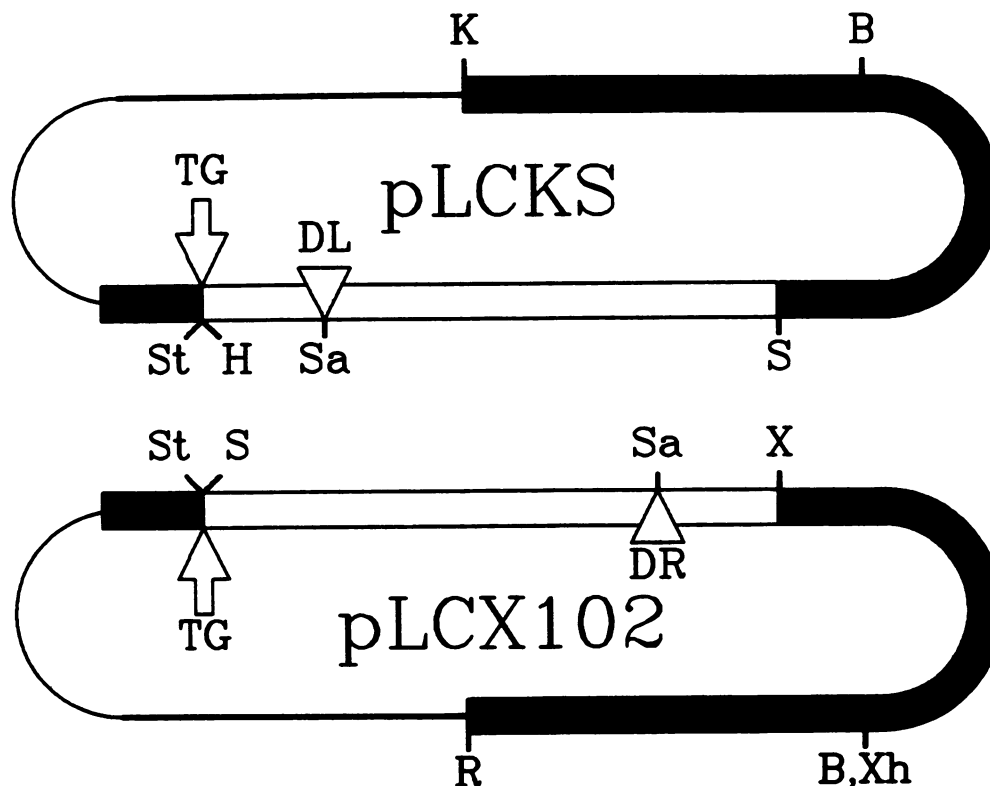


FIG. 1. Circular maps of recombination substrates pLCKS (DL) and pLCX102 (DR). An intermolecular reciprocal exchange event in the region between the two deletions or a gene conversion event that corrects either deletion can give rise to a wild-type *neo* gene. Open box, *neo* gene; TG, insertion points of Z-DNA motif d(TG)<sub>30</sub>; DL, position of deletion left; DR, position of deletion right; shaded box, SV40 origin of replication and early promoter; black box, SV40 3' poly(A) addition signals; line, pBR322 sequences; St, *Stu*I; H, *Hind*III; S, *Sma*I; Sa, *Sal*I; X, *Xba*I; B, *Bam*HI; Xh, *Xho*I; R, *Eco*RI; K, *Kpn*I.

pLCX102 (DR) are derivatives of the procaryotic-eucaryotic shuttle vector pSV2neo that contain deletions of 248 bp of 5' and 283 bp of 3' *neo* coding region, respectively (Fig. 1) (22). There are 501 bp of homology between the sites of the two deletions. Homologous recombination events between the DL and DR molecules, within the interval between the deletions, can give rise to a wild-type *neo* gene. The two deletion plasmids were mixed and introduced into COS-1 cells by DEAE-dextran transfection. After 48 h of replication, low-*M<sub>w</sub>* DNA was isolated and introduced into *E. coli* DH-5, which acts as a reporter system for homologous recombination events that had occurred in the mammalian cells. A sample of the transformed bacterial cells was plated with ampicillin selection to determine the total number of plasmids isolated, and the remaining cells were plated with kanamycin selection to determine the number of *neo*<sup>+</sup> plasmids.

**Homologous recombination occurs in COS-1 cells.** To determine whether recombination was occurring in COS-1 cells, we cointroduced DL and DR molecules into the cells and calculated the Kan<sup>r</sup>/Amp<sup>r</sup> frequency of low-*M<sub>w</sub>* DNA isolated 48 h later. Cotransfection of DL with DR resulted in a  $7.5 \times 10^{-3}$  frequency of Kan<sup>r</sup>/Amp<sup>r</sup> colonies (Table 1), consistent with previous studies (1, 33). Because both of the substrate molecules contain deletions within the *neo* gene, the only way that Kan<sup>r</sup> colonies may be generated is via a homologous recombination event. Although the DH-5 bacterial cells are *recA* mutants and deficient in most homologous recombinations, they do contain other pathways for recombination and could conceivably be contributing to the

generation of Kan<sup>r</sup> molecules. To address that possibility, we introduced DL and DR individually into COS-1 cells, isolated DNA 48 h later, and pooled the two DNA fractions prior to introduction into the bacterial cells. The resulting Kan<sup>r</sup>/Amp<sup>r</sup> frequency was more than 100-fold lower than that determined for cotransfections, indicating that the wild-type *neo* gene was generated in the COS-1 cells and not in the reporter bacterial cells. This observation has been confirmed by several other techniques (1, 35).

**Effects of d(TG)<sub>30</sub> upon homologous recombination in COS-1 cells.** To ascertain the effects of the Z-DNA motif upon homologous recombination between replicating plasmids, we introduced a fragment of DNA bearing d(TG)<sub>30</sub> into two deletion plasmids, pLCKS and pDRD86, between the *Stu*I and *Hind*III sites, to generate the recombination substrates DLTG and DRTG, respectively. The recombination frequency data generated by cotransfection of DLTG and DRTG are presented in Table 1. Surprisingly, the presence of d(TG)<sub>30</sub> in both substrates, which results in a 20-fold stimulation of recombination when introduced into human cells (49), did not stimulate the homologous recombination frequency in COS-1 cells. In fact, the Kan<sup>r</sup>/Amp<sup>r</sup> ratios for the DLTG × DRTG crosses were always lower than those generated by the unsubstituted plasmids, resulting in an overall recombination frequency of  $4.7 \times 10^{-3}$ , or 63% of that observed for the unsubstituted cross. These observations appeared to be in conflict with previous studies which have shown that d(TG)<sub>*n*</sub> stimulates homologous recombination within replicating SV40 viruses from three- to eightfold

TABLE 1. Recombination frequencies between deletion plasmids replicating in COS cells

Input DNA <sup>a</sup>	No. of expts	Kan <sup>r</sup> /Amp <sup>r</sup> colonies <sup>b</sup>	Recombination frequency (10 <sup>-3</sup> )	Ratio
DL + DR (separate transfections) <sup>c</sup>	3	0/48,000	<0.02	
DL + DRTG (separate transfections) <sup>c</sup>	3	0/52,250	<0.02	
DL + DR	5	781/103,698	7.5	1.00
DLTG + DRTG	4	152/32,604	4.7	0.63
DL $\delta$ SH + DR $\delta$ SH	4	363/93,476	3.9	1.00 (0.52) <sup>d</sup>
DLTG + DR $\delta$ SH	4	211/38,259	5.5	1.41
DL $\delta$ SH + DRTG	4	159/43,672	3.6	0.92
DLTG + DRTG	4	657/102,680	6.4	1.64

<sup>a</sup> A 10- $\mu$ g portion of each substrate DNA was introduced into  $2 \times 10^6$  COS cells per 100-mm plate in each experiment.

<sup>b</sup> Transformation of bacteria resulted in 1 ml of culture. A 10- $\mu$ l portion of this culture was used to deduce the total number of Amp<sup>r</sup> colony-forming units. The remaining culture was used to determine the number of Kan<sup>r</sup> colony-forming units.

<sup>c</sup> Recombination substrates were individually transfected into COS cells. Low- $M_w$  DNA isolated from each transfection was mixed together and used to transform *recA* mutant *E. coli*.

<sup>d</sup> Ratio of recombination relative to that between unmodified substrates containing intact T-ag-binding site.

(2, 42); however, the configuration of our assay system offers an alternative explanation for this discrepancy.

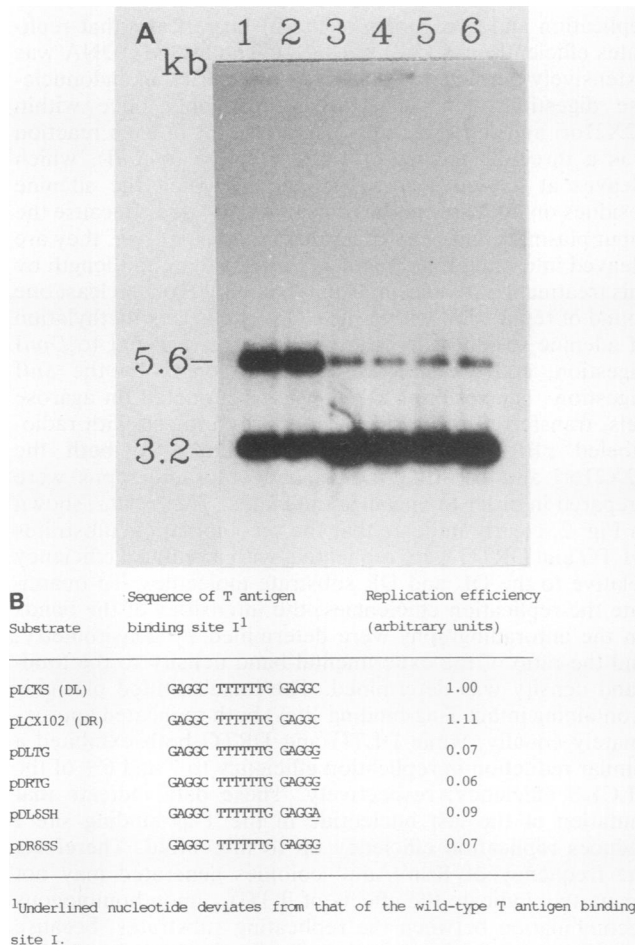
We chose to introduce the fragment of DNA containing d(TG)<sub>30</sub> between the *StuI* and *HindIII* sites of our recombination substrates for two reasons. First, although d(TG)<sub>n</sub> has been shown to stimulate exchange events within adjacent DNA sequences (2), we were unsure whether they could stimulate recombination at large distances. It was, therefore, desirable to place them as near the selected interval (i.e., the region between the two deletions in which recombination must occur to generate a wild-type *neo* gene) as possible and yet outside of the *neo* coding region to avoid generating additional mutations above those created by the deletions. Second, the presence of the *StuI* site adjacent to the *HindIII* site facilitated directional cloning of the fragment which would not have been available had we inserted the fragment into the *SmaI* site 3' of the *neo* gene. Thus, the d(TG)<sub>30</sub> sequence is inserted immediately 5' of the *neo* gene, 237 bp away from the site of the nearest deletion. A possible problem with these constructs is demonstrated in Fig. 2. Whereas the DL and DR plasmids both contain a complete T-ag-binding site I, the DLTG and DRTG substrates contain binding sites in which the last nucleotide residue has been altered from a cytidine to a guanine. Plasmids containing similar mutations are capable of T-ag-dependent replication, but at a reduced level (36). Should DLTG and DRTG be replicating at a significantly lower efficiency than their unsubstituted counterparts, this would result in a lower effective concentration of substrate molecules and could, correspondingly, reduce the apparent recombination frequency. To determine whether the substrate concentrations were similar, we conducted transient replication assays.

**Replication efficiencies of recombination substrates.** An established replication assay was used to determine the relative replication efficiencies of the recombination substrates (3, 24, 47, 52). COS-1 cells were cotransfected with substrate DNA and p2X21ori and incubated for 56 h to allow replication to occur, and low- $M_w$  DNA was isolated. p2X21ori is a small plasmid, containing the SV40 origin of

replication and two copies of the 21-bp repeats, that replicates efficiently in COS-1 cells (24). The low- $M_w$  DNA was extensively purified and linearized by restriction endonuclease digestion with *SalI*, which cuts only once within p2X21ori and the substrates. Also present in each reaction was a threefold excess of *DpnI* (relative to *SalI*), which cleaves at a 4-bp recognition site in which the adenine residues on both strands have been methylated. Because the input plasmids had been generated in *dam*<sup>+</sup> *E. coli*, they are cleaved into numerous fragments of less than unit length by this treatment. Any plasmids that had undergone at least one round of replication within the COS-1 cells lack methylation of adenine residues on both strands, are resistant to *DpnI* digestion, and are linearized to unit length by the *SalI* digestion. The resulting DNA was fractionated on agarose gels, transferred to membranes, and hybridized with radio-labeled pBR322 probe, which hybridizes to both the p2X21ori and substrate DNA, and autoradiographs were prepared in order to visualize the bands. These data, shown in Fig. 2, clearly indicate that the recombination substrates DLTG and DRTG were replicating with a reduced efficiency relative to the DL and DR substrate molecules. To quantify the replication efficiencies, the intensities of the bands on the autoradiographs were determined by densitometry, and the ratio of the experimental-band density to the total-band density was determined. The unsubstituted plasmids (containing intact T-ag-binding sites) both replicated approximately equally, while DLTG and DRTG both exhibited a similar reduction in replication efficiency to 7 and 6% of the pLCKS efficiency, respectively. These data indicate that mutation of the last nucleotide in the T-ag-binding site I reduces replication efficiency up to fifteenfold. Therefore, the frequency of Kan<sup>r</sup>/Amp<sup>r</sup> colonies generated may not accurately indicate the effects of d(TG)<sub>30</sub> upon homologous recombination between the replicating substrates, because the effective substrate concentration in the DLTG  $\times$  DRTG cross is lower than in the DL  $\times$  DR cross.

**d(TG)<sub>30</sub> fails to stimulate recombination between substrates with adjusted replication efficiencies.** In order to accurately determine the effects of d(TG)<sub>30</sub> upon homologous recombination between replicating substrate molecules in COS-1 cells, it was first necessary to adjust the replication efficiencies of the substrates to an equivalent level. This was accomplished by removing the *StuI-HindIII* fragment from pLCKS and the *StuI-SmaI* fragment from pLCX102, filling in the recessed 3' *HindIII* end, and religating the molecules to generate the modified deletion plasmids DL $\delta$ SH and DR $\delta$ SS. Sequence analysis of the two constructs confirmed that the cytosine residue in the last position of T-ag-binding site I had been converted to an adenine in DL $\delta$ SH and to a guanine in DL $\delta$ SS (data not shown). These substrates were subject to the replication assay and found to be capable of replication, but at a reduced level. The replication efficiencies of DL $\delta$ SH and DR $\delta$ SS were equivalent to those of DLTG and DRTG (Fig. 2).

Once the replication efficiencies had been equalized, we cotransfected COS-1 cells with various combinations of the modified unsubstituted deletion plasmids or deletion plasmids bearing d(TG)<sub>30</sub>. Because there is a positional bias to d(TG)<sub>n</sub> crosses (49), we introduced all four possible substrate combinations into the cells and isolated low- $M_w$  DNA after 48 h to determine the Kan<sup>r</sup>/Amp<sup>r</sup> ratios. The data are presented in Table 1. The recombination frequency between DL $\delta$ SH and DR $\delta$ SS was  $3.9 \times 10^{-3}$ , or 52% of that determined between the unmodified DL and DR substrates, confirming that lowering the substrate concentration reduces



**FIG. 2.** Replication efficiencies of recombination substrates. (A) Autoradiograph of replicated DNA isolated from COS-1 cells. The cells were cotransfected with internal replication control plasmid p2X21ori and recombination substrate plasmid. After 56 h of replication, the low- $M_w$  DNA was isolated and linearized by *Sall* digestion, and the nonreplicated plasmids were cleaved into numerous fragments by incubation with a threefold excess of *DpnI* (relative to *Sall*). The resulting DNA fragments were fractionated on a 0.7% agarose gel, transferred to a membrane, and probed with random-primed pBR322 DNA. Lanes: 1, pLCKS (DL); 2, pLCX102 (DR); 3, DLTG; 4, DRTG; 5, DL $\delta$ SH; 6, DR $\delta$ SS. The internal control plasmid p2X21ori yielded a 3.2-kilobase band, while the recombination substrates all gave rise to a bands of approximately 5.6 kilobases. (B) Sequence of T-ag-binding site I and relative replication efficiencies of the recombination substrates. Cloning of d(TG)<sub>30</sub> into the *StuI* site of the recombination substrates altered the last nucleotide of T-ag-binding site I. The substrates DL $\delta$ SH and DR $\delta$ SS, without the d(TG)<sub>30</sub> insert, were constructed to reduce the replication efficiencies so that they approximate those of DLTG and DRTG. The replication efficiency of each substrate molecule was determined by laser densitometry of the autoradiograph and is expressed in arbitrary units of substrate band intensity relative to the total intensity of both bands.

the recombination frequency. Regardless of whether d(TG)<sub>30</sub> was placed in the DL, DR, or in both substrate molecules introduced into the COS-1 cells, there was no significant alteration of recombination frequencies. The failure of d(TG)<sub>30</sub> to stimulate recombination in those experiments indicated that efficiency of substrate replication was not a major factor and that some other aspect of the system was responsible. Two main possibilities exist. First, some aspect

of replication of our substrate molecules, either enzymatic or pertaining to the conformation of the replicating substrates, is abrogating the stimulation that we observe between nonreplicating molecules containing d(TG)<sub>30</sub>. Second, it is possible that the simian cells fail to produce a recombinase that interacts with d(TG)<sub>n</sub>, or that they are producing a factor that interferes with the interaction of such a recombinase. Both of these possibilities are somewhat at odds with previous observations because it has been demonstrated that d(TG)<sub>n</sub> does stimulate recombination within replicating substrates in COS-1 cells (2, 42). In order to distinguish between the possibilities, we analyzed the ability of d(TG)<sub>30</sub> to promote homologous recombination between nonreplicating substrate molecules in the simian cell line CV-1.

**d(TG)<sub>30</sub> stimulates homologous recombination between nonreplicating substrates in simian cells.** The cell line CV-1 is the parental cell line of COS-1 cells and, because it lacks T-ag production, provides a similar phenotypic background in which to assay the effects of d(TG)<sub>30</sub> upon homologous recombination between nonreplicating substrates in simian cells. The assay for homologous recombination between nonreplicating substrates was as previously described (22, 49). The two deletion molecules DL and DR were introduced into the cells by calcium phosphate precipitation. In a certain proportion of the cells homologous recombination occurred between the DL and DR molecules to reconstitute a wild-type *neo* gene. The plasmids then integrated randomly into the chromosome and, after a period of time in selective medium, gave rise to G418<sup>r</sup> colonies. The recombination frequency was calculated as the number of G418<sup>r</sup> colonies generated per microgram of input deletion plasmid DNA divided by the number of G418<sup>r</sup> colonies generated per microgram of wild-type pSV2neo.

We cotransfected CV-1 cells with either DL and DR or DLTG and DRTG and, after selection, stained the resulting colonies and calculated the recombination frequencies presented in Table 2. The results were essentially the same as those determined in human cells (49). The presence of d(TG)<sub>30</sub> in the wild-type pSV2neo plasmid did not significantly alter the frequency of transfection. Homologous recombination occurred between the DL and DR molecules at a frequency of  $1.5 \times 10^{-3}$ , and the presence of d(TG)<sub>30</sub> in both substrate molecules stimulated that recombination thirteenfold. These observations indicate that simian cells do contain the necessary factors for d(TG)<sub>n</sub>-promoted recombination and that the failure to observe stimulation of recombination in the COS-1 cells stems from some aspect of the replication of the recombination substrates.

**T-ag abrogates d(TG)<sub>30</sub>-mediated stimulation of recombination between nonreplicating substrates.** Because the d(TG)<sub>30</sub> sequence was cloned immediately adjacent to the T-ag-binding site I, it is possible that binding of the T-ag could be responsible for the abrogation of d(TG)<sub>30</sub>-stimulated recombination. Alternatively, the conformation of the replicating molecules could be responsible. To distinguish between these two possibilities, we determined recombination frequencies in the pSVCT3-transformed human cell line CCD156. These cells actively produce T-ag with a mutation of amino acid 128 and, although the mutant T-ag can bind to binding site I, do not support the replication of plasmids containing an SV40 origin of replication (4). The data are presented in Table 2. CCD156 cells were found to be capable of catalyzing homologous recombination between the DL and DR substrates slightly more efficiently than CV1 cells were; however, the presence of d(TG)<sub>30</sub> resulted in no stimulation of recombination. This observation, in view of

TABLE 2. Recombination frequencies between nonreplicating deletion plasmids

Cell line <sup>a</sup>	Input DNA <sup>b</sup>	No. of expts	Total colonies/total $\mu\text{g}$ of DNA	Colonies/ $\mu\text{g}$ of DNA	Recombination frequency ( $10^{-3}$ ) <sup>c</sup>	Enhancement ratio <sup>d</sup>
CV-1	pSV2neo	4	515/8	64.38		
	pSV2neoTG	4	441/8	55.13		
	DL + DR	4	19/200	0.095	1.48	1.00
	DLTG + DRTG	4	254/200	1.270	19.73	13.33
CCD156	pSV2neo	4	335/4	83.75		
	pSV2neoTG	4	306/4	76.50		
	DL + DR	4	46/200	0.230	2.75	1.00
	DLTG + DRTG	4	57/200	0.285	3.40	1.24

<sup>a</sup> CV-1 cells do not produce SV40 large T-ag; CCD156 cells have been transformed with pSVCT3 and constitutively produce SV40 large T-ag.

<sup>b</sup> In each experiment, 1  $\mu\text{g}$  of wild-type DNA or 10  $\mu\text{g}$  of each substrate DNA was presented per  $10^6$  cells on a 60-mm dish.

<sup>c</sup> Calculated as (G418<sup>r</sup> colonies/ $\mu\text{g}$  of DL + DR)/(G418<sup>r</sup> colonies/ $\mu\text{g}$  of pSV2neo).

<sup>d</sup> Ratio of the recombination frequency of any substrate combination relative to the recombination frequency of the unsubstituted DL  $\times$  DR cross.

those previously described, indicates that the binding of SV40 T-ag immediately adjacent to the d(TG)<sub>30</sub> sequence abrogates the recombinogenic effects of the Z-DNA motif.

### DISCUSSION

We have shown that d(TG)<sub>30</sub> adjacent to a T-ag-binding site I can stimulate homologous recombination between nonreplicating plasmids, providing that T-ag is absent, in both human EJ and monkey CV-1 cells. It has also been shown elsewhere that the presence of d(TG)<sub>n</sub> not adjacent to the T-ag-binding site can stimulate homologous recombination in SV40 molecules replicating in the presence of T-ag (2, 42). However, we have demonstrated in this study that d(TG)<sub>30</sub> adjacent to a T-ag-binding site bound with T-ag does not stimulate recombination in either replicating or nonreplicating systems. The adjacently bound T-ag, therefore, in some way prevents the recombinogenic activity of the d(TG)<sub>30</sub> sequence.

In our system, the d(TG)<sub>30</sub> sequence begins nine bp away from T-ag-binding site I, and the selected interval (i.e., the *neo* gene) is located on the opposite side of the Z-DNA tract from the T-ag-binding site I. Although it is unlikely that the T-ag is directly interacting with the Z-DNA motif, its binding to the adjacent origin of replication could abrogate the recombinogenic activity of d(TG)<sub>30</sub> in either of two ways. (i) The bound T-ag could prevent the d(TG)<sub>30</sub> sequence from adopting the left-handed Z-DNA conformation. (ii) The bound T-ag may interfere with the binding of specific strand exchange enzymes required for the recombinogenic action of d(TG)<sub>30</sub>. Consistent with these proposals are two major activities of T-ag site-specific binding and a DNA helicase activity. After T-ag has bound specifically to binding sites I and II of the replication origin (8, 9, 46), it carries out an origin-dependent unwinding activity in the presence of ATP, MgCl<sub>2</sub>, and single-strand-binding protein (5, 51). Furthermore the T-ag contains an intrinsic DNA helicase activity that can unwind duplex DNA distal to its binding sites (6, 41, 50). Thus, the origin localization, helix opening, and helicase activities are all provided solely by the T-ag, and, subsequently, other host factors are required to complete the replication process (reviewed in reference 18). Therefore, the protein-DNA preelongation complex corresponds to a partially unwound DNA molecule stabilized by a single-strand-binding protein (7), and the unwound regions may be removing Z-DNA structure from the d(TG)<sub>30</sub> sequence that is required for the sequence to promote homologous recombination. Alternatively, the bound T-ag may prevent the binding of a specific recombinase to the d(TG)<sub>30</sub> sequence.

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