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Effects of Poly[d(pGpT) · d(pApC)] and Poly[d(pCpG) · d(pCpG)] Repeats on Homologous Recombination in Somatic Cells

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Sequencing studies have shown that in somatic cells alternating runs of purines and pyrimidines are frequently associated with recombination crossover points. To test whether such sequences actually promote recombination, we have examined the effects of poly[d(pGpT) · d(pApC)] and poly[d(pCpG) · d(pCpG)] repeats on a homologous recombination event. The parental molecule used in this study, pSVLD, is capable of generating wild-type simian virus 40 DNA via recombination across two 751-base-pair regions of homology and has been described previously (Miller et al., Proc. Natl. Acad. Sci. USA 81:7534–7538, 1984). Single inserts of either a poly[d(pGpT) · d(pApC)] repeat or a poly[d(pCpG) · d(pCpG)] repeat were positioned adjacent to one region of homology in such a way that the recombination product, wild-type simian virus 40 DNA, could be formed only by recombination within the homologies and not by recombination across the alternating purine-pyrimidine repeats. We have found that upon transfection of test DNAs into simian cells, a poly[d(pCpG) · d(pCpG)] repeat enhanced homologous recombination 10- to 15-fold, whereas a poly[d(pGpT) · d(pApC)] repeat had less effect. These results are discussed in terms of the features of these repeats that might be responsible for promoting homologous recombination.

Simian virus 40 (SV40)-transformed cell lines, SV40 viral DNA, and SV40-based recombinant DNA vectors have been used to study both nonhomologous and homologous recombination in somatic cells (4–8, 31, 36). One outcome of these studies has been the observation that the two known genomic crossover sites for SV40 integration are associated with stretches of alternating purines and pyrimidines (8, 41). Moreover, it has been reported that an SV40 genome containing a stretch of alternating purines and pyrimidines, poly[d(pGpT) · d(pApC)] [poly(GT)], undergoes homologous recombination within the alternating purines and pyrimidines up to eight times more frequently than a control insert of the same length (43). However, though alternating purine-pyrimidine repeats may enhance the frequency of certain recombination events involving viral DNA, they are clearly not manditory. For instance, not all of the genomic integration sites utilized by DNA tumor viruses are associated with alternating purines and pyrimidines. Hayday et al. (16) and Williams and Fried (51a) have found that these sequences are not present at the two known genomic sites selected for use during polyomavirus integration.

Additional evidence, often circumstantial, linking various runs of alternating purines and pyrimidines with recombination events has been obtained from other eucaryotic recombination systems (11, 19, 37, 38), and a review of the data linking poly(GT) sequences with recombination has been published (35). Eucaryotic organisms contain many such poly(GT) repeats dispersed throughout their genomes, ranging in size from 10 to 50 base pairs (bp) (14, 15). However, unlike poly(GT) repeats, large poly[d(pCpG) · d(pCpG)] [poly(CG)] repeats have not been found in higher organisms, although fragments of DNA have been identified in which CpG sequences are highly clustered (3). The original proposal that alternating purines and pyrimidines were hot spots for DNA exchanges was made by Slightom et al. (39), after finding a run of alternating poly(GT) residues at one end of a segment of DNA that was suggested to have recombined during duplication of the human G_γ and A_γ globin genes. At present, the mechanism(s) by which runs of alternating purines and pyrimidines might promote recombination in vivo is unknown.

In light of these studies, we decided to test whether poly(CG) or poly(GT) residues could be shown to promote recombination in vivo. In this paper, we present studies of the effects of these two simple sequences on an intramolecular homologous recombination event between two 751-base-pair (bp) direct repeats of SV40. Recombination between the direct repeats generates wild-type (wt) SV40. Since the alternating purines and pyrimidines were cloned precisely at the border of one of the two 751-bp direct repeats, the ability of the alternating purines and pyrimidines to promote recombination in simian cells was easily assessed by following the formation of either SV40 DNA or SV40 virus.

MATERIALS AND METHODS

Plasmid construction and isolation. The construction of plasmid pSVLD was as described previously (31). Plasmid pSVLD-CG_{64} was constructed as follows: plasmid pLP32, which has a 32-bp segment of alternating guanine and cytosine residues cloned into the *Bam*HI site of pBR322 (34), was cleaved with *Bam*HI. After digestion and fragment isolation, the 32-bp poly(CG) insert from pLP32 was ligated into a *Bam*HI partial digestion of pSVLD. A plasmid (pSVLD-GC_{64}) containing a dimer of the poly(CG) insert at the *Bam*HI site that defines the pBR322-SV40 junction in pSVLD was identified by restriction mapping (P. Bullock, Ph.D. thesis, University of California, Berkeley, 1984) and DNA sequencing (data not shown).

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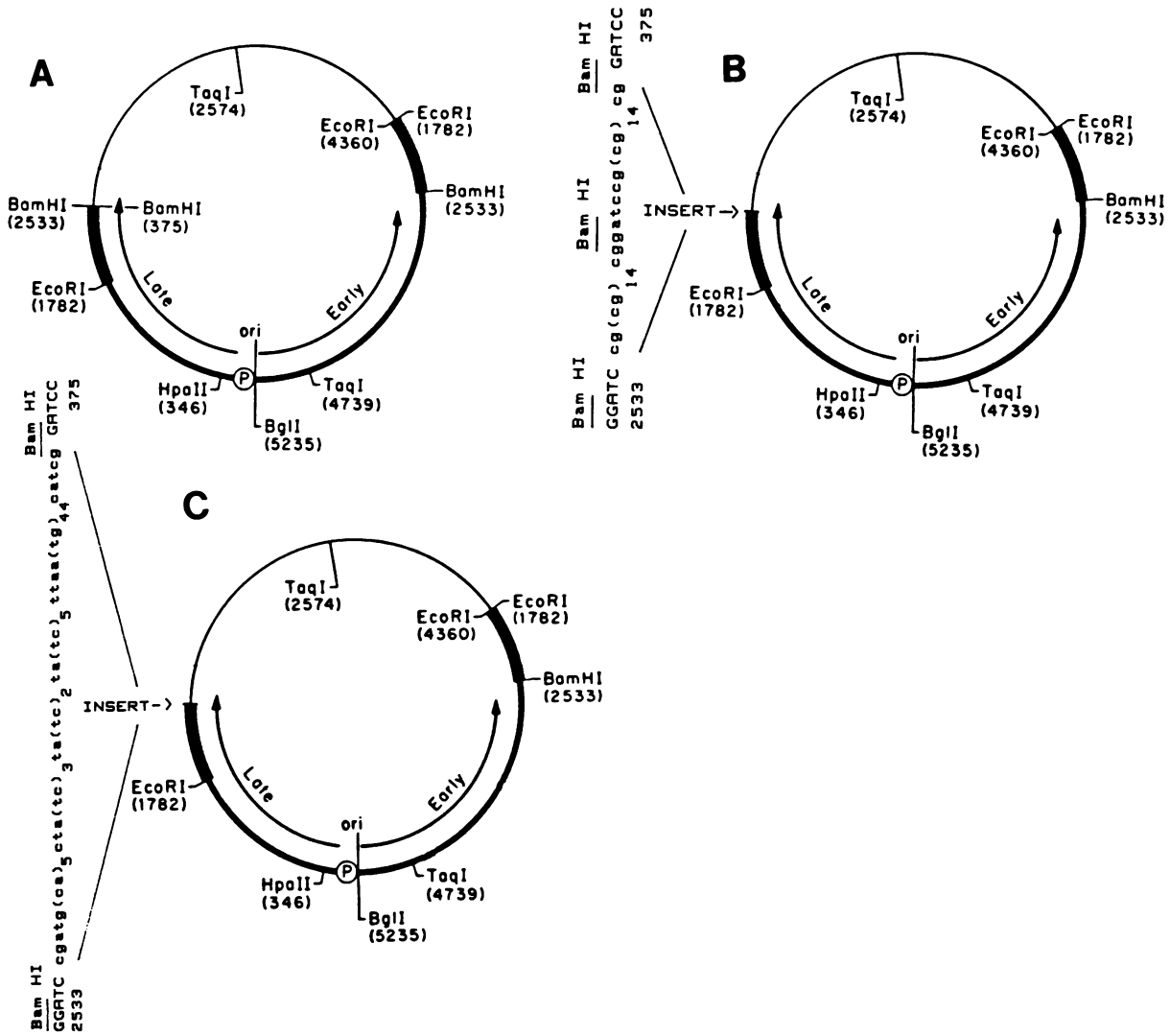

FIG. 1. Structure of plasmids pSVLD (A), pSVLD-CG_{64} (B), and pSVLD-GT (C). All plasmids contain SV40 sequences (thick lines) joined to pBR322 sequences (thin lines). Homologous recombination across the direct 751-bp repeats of SV40 (dark rectangles) reconstitutes wt SV40 DNA. SV40 residues are numbered as described by Tooze (47), and the pBR322 numbering system is that of Sutcliff (45). The nucleotide sequence of the alternating purine and pyrimidine inserts cloned into plasmids pSVLD-GT and pSVLD-CG_{64} (small type) are also indicated.

The source of the poly(GT) repeat in pSVLD-GT was the *Cla*I fragment from plasmid pGT (a gift from J. Stringer). The poly(GT) repeat in plasmid pGT was obtained from Boehringer Mannheim Biochemicals; the sequence of the insert is not completely homogeneous (see below). The *Cla*I sites on the 139-bp poly(GT)-containing fragment were filled in by Klenow fragment-mediated repair, and this piece of DNA was then blunt end ligated into a *Bam*HI partial digestion of pSVLD in which the *Bam*HI sites had also been filled in. A plasmid containing the poly(GT) insert at the *Bam*HI site that defines the pBR322-SV40 junction in pSVLD was identified by restriction mapping (Bullock, thesis), and the orientation of the insert was established by DNA sequencing (data not shown). Plasmid DNAs were isolated by standard procedures (26) and further purified by two cesium chloride and one sucrose gradient ultracentrifugations.

DNA sequencing. The orientation and sequence of the inserts cloned into pSVLD-CG_{64} and pSVLD-GT were established by Maxam and Gilbert sequencing reactions (27) with the modifications of Bencini et al. (2). In both instances, plasmid DNAs were cleaved at the pBR322 *Nar*I site at position 413 and labeled at the 3′ end by Klenow fragment-mediated repair, and asymmetric ends were generated by cleavage of the SV40 *Eco*RI site at position 1782. Confirmation of the nucleotide sequence of the insert cloned into pSVLD-CG_{64} required formation of 5,6 dihydro-6-sulfomethoxycytosine derivatives as described previously (1). This step was necessary to avoid compression of bands within the 8% polyacrylamide sequences gels. To improve our yield, butanol—rather than ethanol—was used to precipitate the cytosine derivatives.

Replication and plaque assays. The SV40 replication assay was essentially that of Lusky and Botchan (25). CV-1 cells for plaque assays (29) were plated onto 60-mm plastic plates and transfected when approximately 95% confluent by the procedure of McCutchen and Pagano (28). DNAs were transfected in 200-μl samples at a DEAE-dextran (Pharmacia Fine Chemicals; molecular weight, 2×10^6) concentration of 500 μg/ml. For plaque assays, cells were

TABLE 1. Relative plaquing efficiency of SV40, pSVLD, pSVLD-CG_{64}, and pSVLD-GT[a]

DNA transfected (ng)	No. of plaques in CV-1 cells			
	SV40	pSVLD	pSVLD-CG_{64}	pSVLD-GT
0.0	0	0	0	0
0.01	2	0	0	0
0.1	26	1 (2)	11 (21)	3 (6)
0.5	107	3 (6)	47 (89)	11 (21)
1.0	~210	7 (13)	~130 (247)	39 (74)
5.0	~400 to 500	26 (49)	~180 (342)	63 (120)
10.0	Confluent	31 (59)	~215 (408)	93 (177)
50.0	Confluent	54 (103)	Confluent	~115 (218)

[a] The recombination potentials of SV40, pSVLD, pSVLD-CG_{64}, and pSVLD-GT were compared by following the formation of infectious SV40 virus upon transfection into CV-1 cells. Plaque assays were performed essentially as described by Mertz and Berg (29). As larger amounts of DNA were transfected, there was less linear response in terms of the number of plaques formed. This interference in SV40 infectivity has been described by Wilson (52). Qualitatively, the same results were obtained from two additional plaque assays. SV40 is 5,243 nucleotides long, whereas pSVLD contains 9,979 nucleotides. Thus, a given mass of SV40 DNA contains roughly twice as many molecules (9,979/5,243 = 1.9) as the same mass of pSVLD. Therefore, the plaques formed by a given mass of pSVLD, or the poly(purine-pyrimidine) derivatives of pSVLD, can be normalized to the number of plaques formed by SV40 by multiplying by 1.9. Plaques normalized to that of SV40 are shown in parentheses.

overlaid with 5 ml of Dulbecco modified Eagle medium–5% fetal calf serum–0.9% Noble agar. Transfected cells were fed after 5 days with 3 ml of agar plus Dulbecco modified Eagle medium containing 1% fetal calf serum. Plaques were visualized on day 10 by including 0.01% neutral red in the final agar overlay.

Enzymes. All enzymes were purchased from standard sources and used according to the manufacturer recommendations.

RESULTS

The structure of the pBR322-SV40 hybrid plasmid pSVLD (SV40 late duplication) is shown in Fig. 1. The usefulness of this plasmid for studying recombination stems from the fact that in this molecule two 751-bp repeats of SV40 sequences are positioned such that recombination across the repeats reconstitutes wt SV40. Thus, upon transfection of pSVLD into permissive simian cells, recombination events can be followed either by using an SV40 replication assay to follow the formation of SV40-sized DNA molecules (25) or by monitoring the appearance of SV40 virus by the plaque assay of Mertz and Berg (29). This type of assay has been used previously by several other groups to study recombination in somatic cells (12, 44, 49). The approach we used for assaying the effects of poly(GT)- or poly(CG)-containing repeats on homologous recombination was to clone these sequences precisely at the border of one of the direct repeats in pSVLD and then, upon transfection of the test plasmids into permissive cells, determine whether these sequences influenced the extent of recombination across the repeats. The steps taken to construct the pSVLD derivatives pSVLD-GC_{64} and pSVLD-GT are described in Materials and Methods, and the structures of these plasmids are shown in Fig. 1.

It is highly improbable that wt SV40 DNA, formed in *Escherichia coli* cells via homologous recombination across the direct repeats, is affecting our results. If formed, the SV40 DNA molecules present in the RecA *E. coli* strain used to propagate these plasmids, DH1, could not replicate. Moreover, SV40 DNA could not be detected when 10-μg samples of plasmids pSVLD, pSVLD-GC_{64}, and pSVLD-GT were analyzed by Southern blots (40) (data not shown). Since picogram quantities of DNA are easily detected by Southern blots, and since 10 pg of wt SV40 DNA gave only two plaques under our experimental conditions (Table 1), the results presented in these studies, with nanogram quantities of plasmid DNAs, do not reflect the presence of preformed wt SV40 DNA.

Determination of the recombination potential of pSVLD and the purine-pyrimidine-containing pSVLD derivatives. The relative recombination potentials of pSVLD and the purine-pyrimidine-containing derivatives of pSVLD were determined via an SV40 replication assay (25). The results of one such experiment are shown in Fig. 2. Control lanes a, b, and c were loaded with 1 ng of pSVLD, pSVLD-CG_{64}, and pSVLD-GT respectively. These parental DNAs contain supercoiled (form I) and nicked-circular (form II) DNA. SV40 DNA, containing forms I and II, was loaded in lane d (~0.1 ng). Lanes e through h contain all of the pSVLD DNAs extracted by the Hirt procedure (17) from 60-mm plates of Cos-7 cells (13) at 0, 20, 40, and 60 h posttransfection. There is little indication that either form I or form II SV40 accumulates in the Cos-7 cells 60 h posttransfection (lane h). The replication products of pSVLD are mainly supercoiled and nicked-circular forms of the parental molecule. However, overexposure of this autoradiogram indicated that a small amount of SV40 DNA (forms I and II) had formed by recombination across the pSVLD direct repeats by 60 h posttransfection. Lanes i through l contain all of the pSVLD-CG_{64} DNA extracted from Cos-7 at 0, 20, 40, and 60 h posttransfection. SV40 DNA was formed by recombination across the direct repeats in pSVLD-CG_{64} (lanes k and l). (A densitometric trace of lane k indicated that approximately 25% of the total DNA isolated at 40 h was either form I or form II SV40 DNA.) Lanes m through p contain all of the pSVLD-GT DNA extracted from Cos-7 cells at 0, 20, 40, and 60 h posttransfection. SV40 DNA is obviously produced within cells transfected with pSVLD-GT (lane p). However, a comparison of either the 40- or 60-h time points indicates that, relative to pSVLD-CG_{64}, less SV40 DNA was produced via recombination across the pSVLD-GT direct repeats. Nevertheless, a comparison of the amount of SV40 (form I and II) present in lane p with that present in lane h indicates that pSVLD-GT promotes recombination more than pSVLD. (A more quantitative measure of this increase is given by the plaque assay in Table 1.) Finally, less of the parental pSVLD-CG_{64} form I DNA is present at 40 h posttransfection than either of the parental form I DNAs from pSVLD or pSVLD-GT (lanes g, k, and o). This result was reproduced in one additional experiment and is more readily seen on lighter exposures of this autoradiogram. The simplest interpretation of this observation is that more pSVLD-CG_{64} DNA has undergone recombination to form SV40 DNA. Collectively these data suggest that, relative to pSVLD, both pSVLD-CG_{64} and pSVLD-GT are preferred

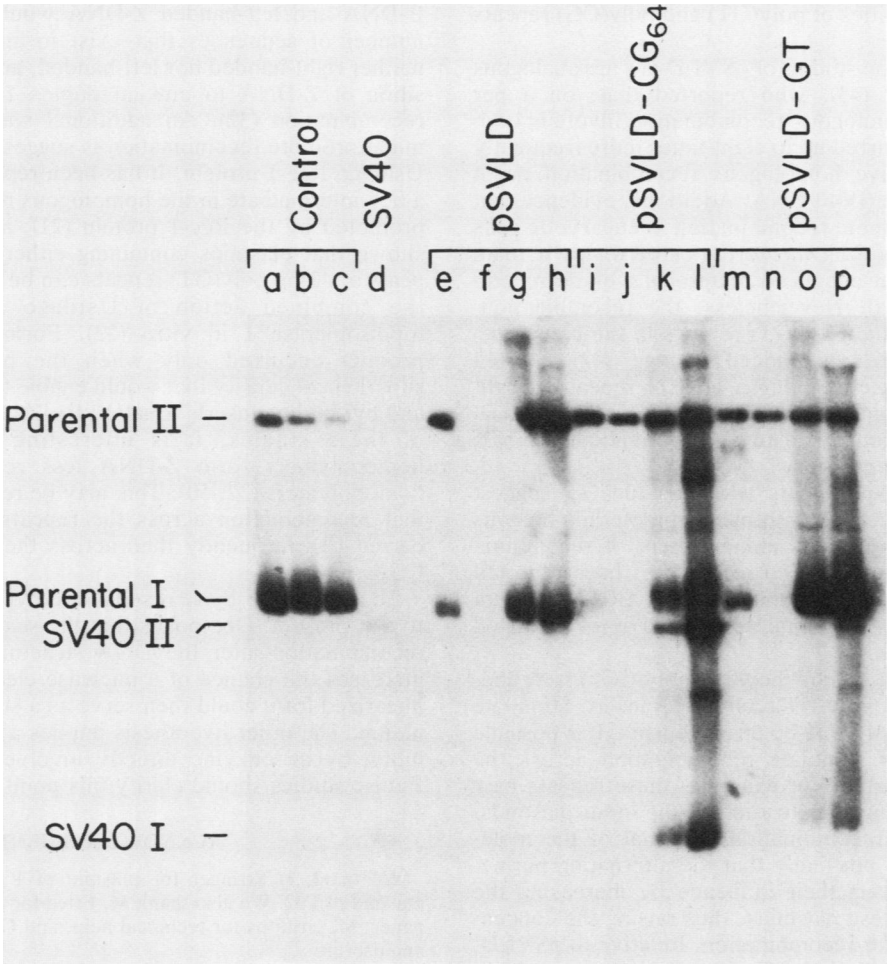

FIG. 2. Detection of SV40 DNA upon transfection of 1 ng of pSVLD, pSVLD-GT, and pSVLD-CG_{64} into Cos-7 cells. Upon transfection into 60-mm plates of Cos-7 cells, low-molecular-weight DNA was isolated by the method of Hirt (17) at 0, 20, 40, and 60 h. Entire isolates, containing supercoiled form I and nicked-circular form II DNAs (some linear form III SV40 DNA, running between SV40 forms I and II, is present in lanes k, l, and p), were fractionated on a 1% agarose gel, transferred to nitrocellulose (40), and probed with nick-translated plasmid pJY1 (25). (Plasmid pJY1 contains the *Bam*HI fragment of SV40 cloned into the pBR322 *Bam*HI site.) Lanes: a through c, approximately 1 ng of pSVLD, pSVLD-CG_{64}, and pSVLD-GT stocks, respectively; d, approximately 0.1 ng of SV40 DNA stock; e through h, low-molecular-weight DNAs extracted from Cos-7 cells transfected with pSVLD at 0, 20, 40, and 60 h; i through l, low-molecular-weight DNAs extracted from Cos-7 cells transfected with pSVLD-CG_{64} at 0, 20, 40, and 60 h; m through p, low-molecular-weight DNAs extracted from Cos-7 cells transfected with pSVLD-GT at 0, 20, 40, and 60 h. In lane f, the DNA did not transfer to the nitrocellulose filter in the vacinity of parental form II pSVLD. Moreover, the apparent mobility of form I SV40 in lanes o and p is high. This distortion was caused by the proximity of the DNA to the edge of the gel.

substrates for recombination, although enhancement of recombination across the direct repeats is greatest in pSVLD-CG_{64}.

This same conclusion was reached by using the plaque assay of Mertz and Berg (29) to follow SV40 virus formation upon transfection of these plasmids into CV-1 cells. Data from one such experiment are presented in Table 1. Recombination across the direct repeats in pSVLD results in a small amount of SV40 virus production (Table 1). However, the number of plaques formed from pSVLD-CG_{64} indicate that a much higher rate of recombination is taking place across the direct repeats in this plasmid. In fact, on a molar basis (Table 1), pSVLD-CG_{64} is almost as effective as SV40 DNA in forming plaques. Moreover, relative to pSVLD, the data from our plaque assay indicate a 10- to 15-fold enhancement of recombination across the 751-bp repeats in pSVLD-CG_{64}. Similar results have been obtained from two additional plaque assays. Finally, the number of plaques formed by pSVLD-GT suggest that the poly(GT)-containing repeat slightly promotes recombination across the repeats (approximately three- to fivefold). This conclusion is supported by the data presented in Fig. 2 and by two additional plaque assays (data not shown).

DISCUSSION

Our experiments demonstrate that certain sequences can promote homologous recombination in somatic cells. We found that the 139-bp poly(GT)-containing insert promoted recombination across the pSVLD direct repeats 3- to 5-fold, whereas the poly(CG) insert increased recombination 10- to 15-fold. However, since 51 bp of nonalternating purine-pyrimidine sequence separated the poly(GT) repeat from the proximal SV40 repeat, a direct comparison of the recombi-

nation-promoting abilities of poly(GT) and poly(CG) repeats cannot be made.

Our findings based on studies of pSVLD-GT are analogous to those of Stringer (43), who reported that, on a per nucleotide basis, homologous recombination involving two poly(GT) repeats occurred up to eight times more frequently than did an alternative homologous recombination event between two tracts of SV40 DNA. Additional evidence that poly(GT) repeats promote recombination in eucaryotic cells comes from studies of *Saccharomyces cerevisiae*, where an 80-bp poly(GT) repeat enhanced reciprocal meiotic recombination fivefold (47a). Nevertheless, the recombination-promoting ability of the poly(GT) repeats in the constructs studied to date is not large. Indeed, Stringer (42) reported that an SV40 variant containing a control repeat of eight tandemly arrayed octamers of the sequence 5'CTCTAGAG3' was more recombinagenic than an SV40 variant containing a poly(GT) at the same position.

In contrast, our experiments with pSV40-CG_{64} suggest that this poly(CG) insert can strongly promote homologous recombination. An analogous enhancement of recombination in procaryotes by poly(CG) repeats has been reported by Klysik et al. (20), who found that poly(CG) repeats of approximately 30 bp or greater promoted *recA*-mediated recombination.

At present, we do not know how the poly(CG) insert and, to a lesser extent the poly(GT)-containing insert, stimulate recombination across the 751-bp direct repeats. It is possible that these sequences stimulate recombination across the 751-bp repeats indirectly. For example, these repeats may influence the subcellular localization of the input plasmids, thereby altering the recombination potential of the molecules. However, it is not likely that the alternating purine-pyrimidine inserts exert their influence by increasing the replication rates of these plasmids, thus raising the concentration of substrates for recombination. Relative to pSVLD, the amount of form I pSVLD-CG_{64} decreases as the amount of form I and II SV40 increases (Fig. 2). This would not be the case if an increase in recombination products (SV40) simply reflected an increase in recombination substrates (pSVLD-CG_{64}).

Alternatively, the alternating purine and pyrimidine repeats may directly stimulate recombination across the repeats owing to either primary or secondary structural features. For instance, alternating purines and pyrimidines may be substrates for sequence-specific nucleases that cause strand scission. It has frequently been observed that breaks in DNA promote recombination (23, 24, 33, 48), and many models of DNA recombination require the existence of such free ends (18, 30, 46). With regard to possible secondary structure considerations, a feature of the poly(CG) insert, although not a feature of the poly(GT) insert, is that these sequences can potentially adopt a cruciform structure that might influence recombination. However, Courey and Wang (9) have presented evidence that, under certain in vitro conditions, cruciform formation is kinetically restricted. Nevertheless, it is not clear what in vivo conditions the biologically active population of pSVLD-CG_{64} molecules is subjected to during transfection or how these conditions might effect cruciform formation. A further possibility is that the recombination enhancement of the alternating purine and pyrimidine inserts is due to the formation of left-handed Z-DNA. Runs of alternating purines and pyrimidines have been reported to be a prerequisite for the formation of Z-DNA in vitro (10, 51). Moreover, it has been proposed that DNA segments in an equilibrium between right-handed B-DNA and left-handed Z-DNA would have a significant number of sequences that exist in an untwisted version, neither right-handed nor left-handed, and that this predisposition of Z-DNA to unwind duplex DNA might promote recombination (32). An additional way in which Z-DNA might promote recombination is suggested by studies of the Ustilago Rec-1 protein. It has been reported that Z-DNA is a key intermediate in the homologous recombination events promoted by the Rec-1 protein (21), and it has now been shown that plasmids containing either 32-bp poly(CG) repeats or 42-bp poly(GT) repeats can be paired and linked by the combined action of Ustilago Rec-1 protein and topoisomerase 1 in vitro (22). Formation of the linked product occurred only when the plasmids were at a superhelical density that would enable the alternating purine and pyrimidine inserts to adopt the Z conformation. In light of these studies, it is interesting to note that G-T homopolymers form Z-DNA less readily than do C-G homopolymers (32, 50). This may be relevant to our finding that recombination across the repeats in pSVLD-GT occurred less frequently than across the repeats in pSVLD-CG_{64}.

To account for these results, two hypotheses seem attractive at present. One posits that the sequences that enhance recombination alter the DNA structure in a manner that increases the chance of a nuclease cleavage. The resulting linearized form could then serve as a substrate for recombination. The other hypothesis implies a more specific recognition by an enzyme directly involved in recombination. Future studies should clarify this point.

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