Rat L (long interspersed repeated DNA) elements contain guanine-rich homopurine sequences that induce unpairing of contiguous duplex DNA

(replication/transposition/non-B-DNA/recombination)

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ABSTRACT The L family (long interspersed repeated DNA) of mobile genetic elements is a persistent feature of the mammalian genome. In rats, this family contains $\approx 40,000$ members and accounts for $\approx 10\%$ of the haploid genome. We demonstrate here that the guanine-rich homopurine stretches located at the right end of L-DNA induce oligonucleotide uptake by contiguous duplex DNA. The uptake is dependent on negative supercoiling and the length of the homopurine stretch and occurs even when the L-DNA homopurine stretches are introduced into a different DNA environment. The bound oligomer primes DNA synthesis when DNA polymerase and deoxyribonucleoside triphosphates are added, resulting in a faithful copy of the template to which the oligonucleotide had bound. The implications of this property of the L-DNA guanine-rich homopurine stretches in the amplification, recombination, and dispersal of L elements is discussed.

Recent work has shown that at least one class of highly repeated mammalian DNA sequences, the mammalian long interspersed DNA family (LINE or L family), which is present in all mammals studied to date (1–3), is a highly amplified collection of transposable elements (4–11). L elements resemble the transposable I elements of *Drosophila* (12), and related DNA elements are also present in other organisms (13, 14). Most rat L elements are full length [6.7 kilobases (kb)], contain a promoter-like sequence at the left end, at least two open reading frames (ORFs), a guanine-rich homopurine (GHP) sequence at the right end, and terminate ≈ 35 base pairs (bp) 3' of the GHP stretches in an adenine-rich region of variable length (Fig. 1) (11).

The mechanism of L-element transposition is not known. Some have suggested that the chromosomal integration of DNA copies of L-DNA transcripts (retroposition) is the cause of L-element amplification (2, 3, 16–18). However, we and others (9, 11) have suggested that a purely DNA-mediated process may also, at least sometimes, be operative. We previously showed (15) that the GHP-containing region at the right end of L elements has two properties that have been implicated in the recombination and replicative amplification of DNA sequences: first, this region can assume a non-B-DNA conformation in negatively supercoiled molecules, as evidenced by its reactivity with bromoacetaldehyde (19); second, the GHP stretches are strong DNA synthesis pause sites for a variety of DNA polymerases *in vitro*. Neither property is exhibited by other regions of L-DNA (15).

Here we show that the GHP sequences induce contiguous non-L duplex DNA sequences to take up complementary single-stranded DNA, which can then prime DNA synthesis

when DNA polymerase and nucleoside triphosphates are added. The uptake of single-stranded DNA and its subsequent priming of *de novo* DNA synthesis are essential intermediates in well-documented models for certain recombinational and transpositional events (20–24). This and other possible effects of the GHP stretches on the recombinational properties of L-DNA are discussed in light of the fact that the presence of GHP stretches at the right end of L elements is an evolutionarily conserved feature of mammalian L-DNA (11, 25, 26).

MATERIALS AND METHODS

Uptake of Polynucleotides and Primer Extension. M13 sequencing primers (–20, –40, and reverse sequencing primer) were purchased from New England Biolabs (nos. 1211, 1212, and 1201, respectively). Phosphorylated (dT)_{25–30} was purchased from Pharmacia and was dephosphorylated with calf intestinal alkaline phosphatase. These oligomers were radiolabeled with ³²P by using [³²P]ATP and T4 polynucleotide kinase according to standard procedures and then purified by chromatography on NENSORB columns (New England Nuclear) as described (15). Plasmid DNA was prepared by alkali lysis (27) and centrifugation through 1 M NaCl. We found, in agreement with the results of others (28), that supercoiled molecules purified by ethidium bromide/cesium chloride centrifugation took up oligonucleotides very poorly. The DNA (100–200 ng) was incubated overnight at 55°C in the case of –20, –40, and reverse sequencing primers, and at 25°C for (dT)_{25–30} in 10 mM Tris-HCl, pH 8.0/50 mM sodium acetate in 10- μ l reaction volumes containing 1×10^4 cpm of radiolabeled primer (≈ 30 pg). The reaction mixtures were then resolved by electrophoresis (100 V for 30 min) in 0.8% agarose gels in a 40 mM Tris acetate buffer (pH 7.9) containing 20 mM sodium acetate. After staining [10 min in ethidium bromide (0.5 μ g/ml)], the gels were dried under vacuum onto DEAE paper (Whatman). Autoradiography was generally for 24–48 hr without an x-ray intensifying screen. Primer extension was carried out with DNA samples prepared by overnight incubation with labeled oligomer as described above and in the legends to Figs. 5–7. The reaction mixtures contained a chemically modified T7 DNA polymerase (29) (“sequenase”) and either all 4 dNTPs (200 μ M) or a mixture of these nucleotides and 2',3'-dideoxythymidine-5'-triphosphate (supplied by United States Biochemical, Cleveland). The primer-extended products were then resolved either by using a denaturing polyacrylamide gel or by alkaline agarose electrophoresis (30) before or after digestion with S1 nuclease.

FIG. 1. Schematic representation of a typical rat L element, based on our previously published DNA sequence of a rat L element called L3 (11) and recent unpublished data on other L-DNA members. Pr, promoter region (heavy solid arrow). The thickened portion of the solid line depicts the region containing the GHP stretches, and the narrow open bar on the right depicts the adenine-rich right end of L-DNA. DNA sequence shown is of the right end of L3 (from the *Ava* II site) and includes the region containing the GHP stretches (nucleotides 1–93), the adenine-rich right end (nucleotides 156–252), and 56 nucleotides of non-L-flanking DNA (target site DNA); the final 12 nucleotides are vector DNA. The adenine-rich right end is boxed, and roman numerals refer to previously demonstrated DNA synthesis pause sites (15). Vd2, Vd3, and UC15, end points of deletions in the GHP stretch (15). Each deletion is fused to the vector sequence shown, which reconstitutes pause site III (Vd3) and a site in the UC15 deletion that is 1 guanine shorter than site II, which we refer to as II' (see Figs. 2 and 4). The A enclosed in a solid box was mutated to a G in deletions Vd2 and Vd3 by *in vitro* mutagenesis (15). The lower case a indicates a weak DNA synthesis pause site observed in Figs. 5 and 6.

RESULTS

We had earlier found that the GHP stretches at the right end of rat L-DNA elements react with bromoacetaldehyde (15), which adds to adenines and cytosines at positions that are normally involved in Watson-Crick base pairs (19). While mapping the reactive nucleotides, we found that although the GHP stretches react most strongly with the reagent, flanking duplex DNA as far as 100 bp also reacted. The reaction of flanking sequences, which has also been reported for sequences 3' of synthetic homoguanine stretches (31), could mean that the L-GHP stretches induce structural alterations in contiguous duplex DNA. Given the nature of the bromoacetaldehyde reaction with DNA, one possibility is that the structural alteration causes the DNA to become either unpaired or susceptible to unpairing.

However, a somewhat trivial explanation for the reaction of bromoacetaldehyde with the flanking DNA is that it is just a secondary effect of the initial major reaction of the reagent with the GHP stretches. This could destabilize the duplex sufficiently to facilitate reaction of the flanking DNA with bromoacetaldehyde.

In the present work, we used an assay for the possible perturbation of flanking DNA by L-GHP stretches that avoids the potential artifacts caused by chemical structural probes. In particular, we determined directly whether L-GHP stretches promote unpairing of flanking DNA by

testing whether the flanking sequences take up complementary oligonucleotides.

Induction of Oligonucleotide Uptake by the GHP Stretch. We first transferred the L-DNA GHP stretches to another sequence environment and examined their effect on adjacent DNA sequences. Fig. 2 shows the clones used, and Fig. 3 (A–C) shows the results of incubating these clones with radiolabeled oligonucleotides A, B, or C that are complementary to pUC19 sequences located either 5' or 3' of the inserts (see Fig. 2). As expected, pUC19 (lanes 1) does not take up any of the oligomers. The L-GHP sequences, however, induce oligonucleotide uptake, and Fig. 3 B and C (lanes 3 and 4) shows that the extent of uptake is dependent on the length of the GHP stretches. Oligonucleotide A is taken up to about the same extent by all three recombinant clones, but the site complementary to this oligonucleotide is 100 bp further away from the GHP region of the UC15 clone than in clones Vd3 and Vd2. These results show that the L-DNA GHP stretches of a certain minimal length induce unpairing of contiguous non-L-DNA.

The effect of the GHP stretches on flanking sequence in these molecules is dependent on negative supercoiling, since the linear form of UC15 did not take up oligonucleotide (see below and Fig. 6A), and ethidium bromide severely reduced oligonucleotide uptake (results not shown). Since relaxed and linear molecules have the same winding number (32), we presume that the minor radioactive band comigrating with the relaxed form of the GHP-containing clones in Fig. 3 is due, at least in part, to oligonucleotide uptake by the supercoiled forms of the concatemers present in the plasmid preparations (see Fig. 3D).

In the case of normal rat L members, the GHP stretches could affect the adenine-rich right end of the element and, if the adenine-rich right end is only 10–20 bp long [as is usual (33)], the non-L target site DNA as well. To determine whether the adenine-rich end could take up oligomer in the presence or absence of GHP stretches, we constructed the clones shown in Fig. 4 (*Upper*). We tested whether these clones take up oligomer by incubating them with dT_{25–30}. Fig. 4B shows that whereas the adenine-rich right end on its own did not take up oligo(dT) (UC19A, lane 1), the presence of the Vd3 sequence (lane 2) or the intact GHP region (clone pF5b, lane 3) induced it to do so. This reaction takes place at 25°C or 37°C and is also supercoil dependent. In other experiments in which 80 times more oligo(dT) was used, clone 19A bound a small amount of oligo(dT) (see Fig. 7). This might be expected since the 19A insert contains an 18-bp imperfect

Fig. 2. pUC19 clones containing various amounts of the L3 GHP region. Clones Vd2, Vd3, and UC15 contain 32, 60, and 85 bp of the DNA 3' of and including the *Ava* II site shown in Fig. 1. A, B, and C and corresponding symbols refer to the sites complementary to the M13 reverse, -20, and -40 sequencing primers, respectively. Heavy solid bars, GHP-containing regions; thin solid lines, pUC19 sequences. Hatched regions, either a pBR322 or M13mp19 sequence. The sites for oligonucleotides B and C are 17 and 38 bp, respectively, 3' of the GHP region. The site for oligonucleotide A is 70 bp 5' of the GHP region in clones Vd2 and Vd3, and 167 bp 5' of the GHP region in clone UC15.

FIG. 3. Oligonucleotide uptake by duplex molecules containing the GHP region. Each of the plasmids shown in Fig. 2 was incubated at 55°C (the calculated optimal hybridization temperature) with radioactive oligonucleotides. Lanes 1–4 contain pUC19, Vd2, Vd3, and UC15, respectively. (A, B, and C) Reaction of plasmids with oligonucleotides A, B, and C (see Fig. 2). (D) Ethidium bromide-stained gel of the experiment shown in B prior to drying and autoradiography. R, relaxed plasmid; S, supercoiled plasmid. In these experiments, primer was in limiting concentration. The same results as shown here were found when primer was in molar excess of plasmid DNA (data not shown).

homopurine stretch that is 50% guanine (see sequence beginning at position 102 in Fig. 1). However, as was found in the experiment shown in Fig. 4B, the GHP stretch in clone Vd3A greatly stimulated the binding of the oligomer in the experiment shown in Fig. 7 (see below).

We note that the extent of oligo(dT) uptake shown in Fig. 4B is about the same whether or not the GHP region is partially deleted (see Fig. 3B and C, lanes 3 and 4). This could be explained in part by the fact that the plasmids used for the oligo(dT) uptake are significantly different in length (Vd3A is 2.7 kb, pF5b is 4.5 kb) and that the effect of negative supercoiling may be greater in smaller molecules than in larger ones. Neither clone Vd3A nor UC19A takes up oligonucleotides B or C (results not shown).

Although supercoiled molecules nonenzymatically take up homologous DNA strands under certain conditions (28), pUC19 did not hybridize any of the oligonucleotides tested (Fig. 3). The relatively low temperature and salt concentration of our reactions (see *Materials and Methods* and legend to Fig. 3) might explain these results. Whatever the explanation, the results of Fig. 3 show that the presence of GHP stretches of a certain minimal length is an absolute requirement for oligonucleotide uptake by pUC19 under our reaction conditions.

Oligonucleotides Taken Up by Supercoiled Molecules Can Prime DNA Synthesis *in Vitro*. The uptake of oligonucleotides by supercoiled DNA presumably results in the formation of a “D loop,” which is a three-stranded structure consisting of

a duplex between the invading oligonucleotide and one strand of the invaded molecule; the third strand of the structure, which is complementary to the sequence that has bound the oligonucleotide, is unpaired. A D loop can serve as a substrate for DNA synthesis and is also an essential intermediate in certain recombinational events. Therefore, if bona fide D loops were formed as a result of the oligonucleotide uptake and if they were sufficient in size to permit entry of DNA polymerase, then we would expect that the oligonucleotides should be extended to produce a faithful copy of the template to which the oligonucleotide hybridized. To test this, we incubated UC15 that had taken up either oligonucleotide B (the –20 M13 sequencing primer) or oligonucleotide C (the –40 M13 sequencing primer) with a chemically modified T7 DNA polymerase (29) as described in *Materials and Methods*. As the “T tracks” in Fig. 5 (lanes 2 and 4) show, the expected products were produced from both primers. Lanes 1 and 3 show that while the modified T7 DNA polymerase pauses at sites II’ and III, as we previously showed for a number of other DNA polymerases by using a single-stranded template containing this region (15), the enzyme polymerizes rather long products on these templates.

To determine whether the newly synthesized DNA remained bound to the template, we examined the products of another reaction, both before and after S1 nuclease digestion. In this experiment, we also examined oligonucleotide uptake

FIG. 4. Uptake of oligo(dT) by clones containing the adenine-rich right end of L3. (Upper) Relevant region of the clones used for the experiment shown below. Each clone contains nucleotides 101–314 in the sequence shown in Fig. 1 (open box). Clone pF5b is a pBR327 derivative containing the entire DNA sequence shown in Fig. 1B plus an additional 1.3 kb of L-DNA extending leftward from the *Ava* II site (see Fig. 1). Heavy solid bars, the GHP region starting with the *Ava* II site shown in Fig. 1. (Lower) Results obtained when 600 ng of UC19A, 100 ng of Vd3A, and 200 ng of pF5b DNA was incubated with radioactive (dT)_{25–30} at 25°C. Lanes 1–3, clones UC19A, Vd3A, and pF5b, respectively. (A) Ethidium bromide-stained gel for the experiment shown in B. Arrowheads, bands corresponding to the supercoiled plasmid DNA.

FIG. 5. Bound oligomer serves as a primer for DNA synthesis. Clone UC15 was incubated with 1 ng each of oligonucleotides B or C as described. Four-tenths of each oligomer–plasmid complex was incubated with chemically modified T7 DNA polymerase (29) together with 4 dNTPs, with or without ddTTP. Lanes: 1 and 2, oligomer B plus UC15, without (lane 1) and with (lane 2) ddTTP; 3 and 4, oligomer C plus UC15, without (lane 3) and with (lane 4) ddTTP. Roman numerals refer to the DNA synthesis pause sites (see Fig. 1 and legend). These pause sites are also observed when the sequence being copied is present as a single-stranded template (ref. 15; data not shown). The numbers 67, 245, and 420 refer to the length of the nucleotide chains migrating at the indicated position. This was determined from the “T track” shown (lanes 2 and 4) as well as from other sequencing reactions that were also applied to this gel (data not shown). Incubation of UC15 with just the T7 DNA polymerase showed no relaxation of the supercoiled plasmid, as judged by gel electrophoresis. a and b correspond to similarly designated bands in Fig. 6.

and primer extension by using UC15 that had been linearized by digestion with a restriction enzyme.

Fig. 6A (lane 4) shows that the linear form of UC15 did not hybridize oligonucleotide. The faint radioactive band in lane 4 migrates with the supercoiled form of the plasmid, a small amount of which escaped restriction. Fig. 6B shows the products synthesized by using the oligonucleotide uptake reactions shown in lanes 3 and 4 of Fig. 6A. Most of the products were 400–700 nucleotides long and resistant to S1 nuclease and therefore bound to the template. However, another band (≈ 530 nucleotides, indicated by *) was produced by S1 nuclease digestion, which indicates that a small portion of the products are sensitive to the nuclease. These products would be produced if the T7 polymerase switched strands during synthesis (34).

Fig. 6 also shows that the amount of DNA synthesized is directly related to the amount of supercoiled-bound oligonucleotide in the reaction (compare lanes 3 and 4 in Fig. 6A with lanes 1 and 2, respectively, in Fig. 6B). Finally, analysis of the products of these reactions (or a third primer-extension reaction) on alkaline agarose gels showed that the longest product synthesized was ≈ 700 nucleotides (data not shown).

The oligo(dT) that was taken up can also be extended by the T7 DNA polymerase. Fig. 7 shows that while only trace amounts of synthetic products were detected with clone 19A (lane 1), substantial amounts of products were synthesized

FIG. 6. Analysis of DNA synthesized in the primer-extension reaction. (A) One microgram of supercoiled UC15 or 500 ng of this plasmid that had been first digested with *Sca* I endonuclease was incubated in a 50- μ l reaction mixture with 1.8 ng of oligonucleotide. (B) One-tenth of each reaction mixture was analyzed for oligonucleotide uptake by agarose gel electrophoresis, and the remainder was used for the reactions shown in B. Lanes: 1 and 2, respective ethidium bromide-stained samples; 3 and 4, oligonucleotide taken up by the supercoiled and linear DNA preparations, respectively. R, L, and S, relaxed, linear, and supercoiled forms, respectively, of the plasmid. (B) Remainder of each of the reactions described in A was incubated with T7 DNA polymerase and all 4 dNTPs, and the products were analyzed under denaturing conditions. Lanes: 1 and 2, no further treatment; 3 and 4, S1 nuclease treatment; 5 and 6, boiled and then treated with S1 nuclease. Lanes 1, 3, and 5 or lanes 2, 4, and 6 contain the products synthesized from the supercoiled or linear plasmids, respectively. Lane M, radiolabeled *Hae* III fragments of ϕ X174 DNA. Bands designated III, a, and b correspond to the similarly designated ones in Fig. 5.

FIG. 7. Bound oligo(dT) serves as a primer for DNA synthesis. Clones 19A and Vd3A (200 ng) (see Fig. 4) were incubated in a 10- μ l reaction mixture with 2.4 ng of oligo(dT) at 25°C. A portion of each reaction mixture (≈ 120 ng of each clone) was then incubated with all 4 dNTPs and the T7 DNA polymerase. Lanes: 1 and 2, products using clones 19A and Vd3A, respectively; M, same as in Fig. 6. The numbers 170 and 185 refer to the length (in nucleotides) of the indicated bands and are the shortest products that accumulated in these reactions.

from the oligo(dT) bound by clone Vd3A (lane 2). This indicates that at least some of the bound oligo(dT) formed D loops. The major products synthesized from Vd3A are not as discrete as but are roughly comparable in length to those synthesized from the UC15 clone (compare bands b and c in Fig. 6B, lanes 1 and 3 with the bracketed bands in Fig. 7, lane 2). The 170- and 185-nucleotide chains are the smallest ones detected (Fig. 7, lane 2) and would be produced if oligo(dT) hybridized to the stretch of adenines beginning at position 226 in Fig. 1 and synthesis paused at sites III and a, respectively (see Fig. 1 and compare with Figs. 5 and 6B).

DISCUSSION

Here we used oligonucleotide uptake as an assay for the perturbation of sequences distal to GHP stretches. By introducing the GHP stretches into a DNA environment that on its own did not take up oligonucleotides, we unambiguously showed that the GHP stretches render duplex DNA both 5' and 3' to the GHP stretches susceptible to invasion by oligonucleotides. In analogous experiments, we also showed that L-GHP stretches greatly stimulate the uptake of oligo(dT) by the adenine-rich right end of an L-DNA element. Since all L members contain an adenine-rich right end, this result indicates that the L-GHP stretches could render the right end of L members susceptible to strand invasion by semihomologous target site DNA (33) or oligonucleotides that are present in animal cell nuclei (35).

The D loops formed by the uptake of the oligonucleotides *in vitro* are stable and large enough to permit extension of the oligonucleotide by added modified T7 DNA polymerase. The amount of DNA synthesized is about the same as that synthesized from template-primer complexes formed from approximately equimolar amounts of the single-strand template (results not presented). Therefore, a substantial fraction of the D loops apparently participates in the primer-extension reaction, which our results indicate begins on negatively supercoiled molecules. Most of the products extended on UC15 are 420–700 nucleotides long and remain bound to the template (resistant to S1 nuclease) presumably as a D loop.

Previous work has shown that GHP stretches of a certain minimal length assume an abnormal (non-B) DNA conformation that is detectable in negatively supercoiled molecules

by a variety of means (15, 19, 31, 36–46). This has suggested to some (31, 41, 42) that base stacking in the homopurine strand may be a primary factor in causing the structural perturbation. The length of the L-GHP stretch is correlated with its ability to induce oligonucleotide uptake by distal DNA sequences (Fig. 3), and also with its reactivity with bromoacetaldehyde in negatively supercoiled molecules and the ability of the homopurine strand to partially arrest DNA synthesis *in vitro* (15). Since the complementary homopyrimidine strand did not arrest DNA synthesis, we also had suggested that the homopurine strand assumes an abnormal structure related to base stacking (15).

Although the presence of GHP stretches at the right end of L elements is an evolutionarily conserved feature of mammalian L families (11, 25, 26), the relevance of these sequences to the biological properties of L-DNA elements remains to be proven. However, GHP stretches render flanking DNA very susceptible to two reactions that are essential intermediates in well-documented models for certain recombinational and transpositional events (20–24): synapsis with single-stranded DNA and extension thereof by DNA synthesis. In addition, unpaired sequences in the vicinity of L-GHP stretches may be quite susceptible to strand scission, generating free ends that are highly recombinogenic (47–49). Therefore, L-GHP sequences may account for the fact that the right end of mammalian L-DNA elements are particularly prone to recombination (9, 11, 50, 51). In addition, under certain conditions, this might lead to preferential replication of L-DNA sequences by a mechanism similar to the recombination-dependent synthesis of T4 bacteriophage DNA (30, 52). Finally, the properties that we have described for L-GHP stretches may also apply to other non-L-GHP stretches that have been found near a number of eukaryotic genes (36–39, 53–57).

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