A Novel, Topologically Constrained DNA Molecule Containing a Double Holliday Junction

DESIGN, SYNTHESIS, AND INITIAL BIOCHEMICAL CHARACTERIZATION

Received for publication, March 28, 2006 Published, JBC Papers in Press, April 10, 2006, DOI 10.1074/jbc.M602933200

Jody L. Plank and Tao-shih Hsieh
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The double Holliday junction (dHJ) is a central intermediate to homologous recombination, but biochemical analysis of the metabolism of this structure has been hindered by the lack of a substrate that adequately replicates the endogenous structure. We have synthesized a novel dHJ substrate that consists of two small, double stranded DNA circles conjoined by two Holliday junctions (HJs). Its biochemical synthesis is based on the production of two pairs of single stranded circles from phagemids, followed by their sequential annealing with reverse gyrase. The sequence between the two HJs is identical on both strands, allowing the HJs to migrate without the generation of unpaired regions of DNA, whereas the distance between the HJs is on the order of gene conversion tracts thus far measured in Drosophila and mouse model systems. The structure of this substrate also provides similar topological constraint as would occur in an endogenous dHJ. Digestion of the dHJ substrate by T7 endonuclease I resolves the substrate into crossover and non-crossover products, as predicted by the Szostak model of double strand break repair. This substrate will greatly facilitate the examination of the mechanism of resolution of double Holliday junctions.

Homologous recombination is an important pathway for the repair of double stranded DNA breaks, the restart of stalled replication forks, and the generation of genetic diversity during meiosis. One of the predicted intermediates of homologous recombination is the reciprocal exchange of single strands of DNA between two homologous sequences across the region of the original break (shown diagrammatically in the column of Fig. 1), a structure termed a double Holliday junction (dHJ) (2, 3). It was postulated that the digestion of this structure by endonucleases could give rise to gene conversion events either with (crossovers, CO) or without (non-crossovers, NCO) the exchange of sequences flanking the original break site (see Fig. 1, left branch).

Since that time, many of the components of the model have been discovered. In yeast, it has been demonstrated that exonucleases resect the 5’-strands of the DNA at a double stranded break to reveal ssDNA with a 3’-end which can invade a homolog or sister chromatid to initiate DNA synthesis (4–6). Work by Newlon, Kleckner, and their respective co-workers (7–9) demonstrated that double Holliday junctions exist in vivo, and subsequently Hunter and Kleckner (10) also demonstrated the existence of a stable single-end invasion intermediate that precedes dHJ formation. Numerous endonucleases (or their activities) have been discovered that specifically bind to and cleave synthetic HJs from organisms ranging from bacteria and viruses to yeast and mammalian systems (11–13). When studied in vitro, these endonucleases can cleave a dHJ substrate to yield both CO and NCO products (this work). However, in both yeast and mouse meiosis the generation of crossover and non-crossover products has been shown to be distinctly different processes, not the differential digestion of a common intermediate (14, 15). One of the proposed mechanisms for the generation of NCO products is the collapse of smaller dHJs by a topoisomerase partnered with a helicase (see Fig. 1, right branch) (16, 17).

Currently, the only dHJ-containing substrates that are available for the biochemical study of these processes are constructed from oligonucleotides (18, 19). Although these substrates certainly replicate the dHJ structure, there are many other characteristics of an endogenous dHJ that these substrates cannot recapitulate. Because of technical constraints in the construction of the oligonucleotide substrates, the structures used for these studies are small, with a very short distance between the two HJs, which are necessarily immobile, unlike an endogenous dHJ. In addition to this, these oligonucleotide substrates cannot recapitulate all of the topological constraints inherent to the endogenous structures.

We have synthesized a substrate that consists of two small, double stranded DNA circles conjoined by two Holliday junctions. The sequence between the two HJs is identical on both strands, allowing the HJs to migrate without the generation of unpaired, single stranded regions of DNA. This substrate is also topologically constrained, as migration of a HJ will generate negative writhe behind the HJ and positive writhe in front. Because these writhes will be generated in two topologically isolated regions, this recapitulates the topological constraints that must be intrinsic to endogenous dHJs. The distance between the two HJs is 165 bp, a distance that is comparable to gene conversion tracts that have been measured thus far in Drosophila (~400 bp) and mouse (<533 bp) models (15, 20, 21). We report here the synthesis of this substrate and show evidence supporting its proposed structure.

EXPERIMENTAL PROCEDURES

The Purification of the Enzymes—Reverse gyrase (22) and Cre recombinase (23) were purified from overproducing strains as described previously.

The Synthesis of the DNA Substrates and Marker Molecules—The plasmids used to synthesize the DHJS were created by ligating four small, oligonucleotide based dsDNA fragments into pBluescript SK+ and pBluescript SK−. These inserts contained the two loxP sites along with the balance of the homologous region of the DHJS (supplemental Fig. 1). Sequence A or B was then cloned into the center of the homol-
Synthesis of a Novel Double Holliday Junction Substrate

RESULTS AND DISCUSSION

Design and Synthesis of the DHJS—The DHJS is created from sequences encoded on four phagemid vectors. Two of these vectors are illustrated in Fig. 2A, step I. The vectors are derivatives of pBluescript SK+ and pBluescript SK−, in which two tandem loxP sites are separated by a sequence that includes either sequence A or B. Sequences A and B were chosen to be ~50% of base composition in GC, with no unusual features predicted. Each contain a BamHI site near the center of the sequence that will be utilized later in this study. The resulting phagemids are pDHJS AN+, pDHJS AN−, pDHJS BN+, and pDHJS BN−. Although sequences in A and B are not homologous, sequences outside of A and B are homologous in these vectors.

Single stranded DNA circles were expressed from each of these phagemids by infecting E. coli harboring the phagemid with the helper phage, M13K07. The ssDNA was purified from the supernatants of these cultures using a modified protocol from Bregeon and Doetsch (25) (Fig. 2A, step II). In parallel reactions, BN+ ssDNA was annealed and linked with AN− (Fig. 2A, step III), and AN+ ssDNA was annealed and linked with BN− using reverse gyrase from the hyperthermophile Archaeoglobus fulgidus. The products of these reactions are relaxed, heterodimeric DNA circles containing two unusual features predicted. Each contain a BamHI site near the center of the sequence that will be utilized later in this study.

Design and Synthesis of the DHJS—The DHJS is created from sequences encoded on four phagemid vectors. Two of these vectors are illustrated in Fig. 2A, step I. The vectors are derivatives of pBluescript SK+ and pBluescript SK−, in which two tandem loxP sites are separated by a sequence that includes either sequence A or B. Sequences A and B were chosen to be ~50% of base composition in GC, with no unusual features predicted. Each contain a BamHI site near the center of the sequence that will be utilized later in this study. The resulting phagemids are pDHJS AN+, pDHJS AN−, pDHJS BN+, and pDHJS BN−. Although sequences in A and B are not homologous, sequences outside of A and B are homologous in these vectors.

Single stranded DNA circles were expressed from each of these phagemids by infecting E. coli harboring the phagemid with the helper phage, M13K07. The ssDNA was purified from the supernatants of these cultures using a modified protocol from Bregeon and Doetsch (25) (Fig. 2A, step II). In parallel reactions, BN+ ssDNA was annealed and linked with AN− (Fig. 2A, step III), and AN+ ssDNA was annealed and linked with BN− using reverse gyrase from the hyperthermophile Archaeoglobus fulgidus. The products of these reactions are relaxed, heterodimeric DNA circles containing two unusual features predicted. Each contain a BamHI site near the center of the sequence that will be utilized later in this study.
FIGURE 2. Synthesis of a topologically constrained double Holliday Junction substrate. A, schematic of synthesis. The substrate is derived from four phagemid vectors, containing tandem loxP sites and differing with either sequence A or B, and with the f1 origin in both the plus and minus orientations for each. For simplicity, reactions for only two of the plasmids are shown (I). Using the helper phage M13K07, ssDNA circles are expressed, purified (II), mixed, and linked using reverse gyrase to yield a large heterodimer (III). The large heterodimer is linearized by digestion with XhoI (IV), and the small heterodimer is excised and circularized utilizing Cre recombinase and the loxP sites.
Synthesis of a Novel Double Holliday Junction Substrate

FIGURE 3. Double digest of the DHJS is consistent with the predicted structure. A, digestion of the DHJS with BamHI and NheI should separate the two HJs, allowing them to spontaneously migrate. The migration of the HJs off the ends of the DNA would result in four linear DNAs, 266, 265, 200, and 150 bp in size. B, the double digest of the DHJS yields the predicted fragments. Digestion of the DHJS by BamHI (lane 2) produces a single band with reduced electrophoretic mobility compared with the undigested DHJS (lane 1). DHJS digested with BamHI and NheI (lane 3) produces four bands, which co-migrate with the double digest of marker A (lane 4) and marker B (lane 5). Either the two circular DNAs linked by dHJ or its linearized derivatives are topologically constrained in between HJs thus allowing topoisomeration, which may account for the appearance of species with more than a single electrophoretic mobility in lanes 1 and 2. A DNA size marker was run in lane 6, with the size of the bands denoted to the right of the gel in base pairs.

flanking the A/B bubble (V). The small heterodimer is gel-purified away from the other reaction products of the Cre reaction and is mixed with the complementary small heterodimer generated from the double digest of markers A and B and are of the predicted sizes (Fig. 3B, compare lane 3 with lanes 4–6). This analysis has been carried out using several other restriction enzymes (Fig. 4 and data not shown), and thus far all of the results have been consistent with the predicted structure of the substrate.

The previous analysis was performed without constraining the migration of the HJs predicted to be in the DHJS. We repeated this analysis while constraining the migration of the HJs with interstrand cross-links that were introduced into the DHJS before digestion with restriction enzymes. The DHJS was cross-linked with psoralen, a chemical that creates interstrand cross-links primarily at TA sequences, once the psoralen-DNA complex is irradiated with long wavelength ultraviolet light (26, 27). This prevents the migration of a HJ beyond the cross-link, and has commonly been used to maintain endogenous HJs in previous studies (7–9). The homologous region of the DHJS contains 12 TA dinucleotides, with 4 of them occurring in pairs within the loxP sequence at the center of the homologous region (Fig. 4A). Repeated TA sequences have been shown to be particularly efficient cross-linking sites (27). Based on this, we predicted that the bulk of the cross-links created within the homologous sequence would be located in the loxP sequence, and chose restriction sites bordering the loxP sequence for the analysis of the cross-linked DHJS.

Psoralen cross-linking of the DHJS, and the subsequent inhibition of HJ migration, will change the electrophoretic pattern of the double digest if the substrate possesses the predicted structure. Without psoralen treatment, digestion of the DHJS with BamHI and XmnI, followed by migration of HJs across the homologous regions, will produce four linear fragments. Three of the four linear molecules are of similar length and co-migrate as a single band ~235 bp in size, with the fourth fragment at 180 bp (Fig. 4B). However, if the substrate is cross-linked within the loxP sequence before the double digest, then two of the three molecules that make up this band would contain a trapped HJ and would be electrophoretically retarded on the gel, diminishing the intensity of the 235-bp band. This is precisely the pattern that is observed when the cross-linked DHJS is digested with BamHI and XmnI (Fig. 4D, compare lane 5 with lane 4). The intensity of the 235-bp band present on the cross-linked DHJS lane is diminished...
relative to the control lane, with the appearance of a new band with retarded migration relative to the linear fragments.

The cross-linked DHJS was also analyzed in a similar fashion by digestion with BamHI and EcoRI. Without cross-linking, the double digest yields four linear fragments after branch migration. Three of the fragments (~200 bp in size are expected to co-migrate during electrophoresis, with the fourth 266-bp fragment migrating as a resolved band (Fig. 4C). With psoralen treatment prior to double digest, the HI trapped by cross-linking within the loxP sequence should involve the unique, resolved band as well as one of the three molecules contained within the heterogeneous band. When the psoralen cross-linked DHJS is digested by BamHI and EcoRI, the homogeneous 266-bp band almost completely disappears, and the intensity of the 200-bp heterogeneous band diminishes relative to the control digest with the appearance of a new band with retarded migration relative to the linear fragments (Fig. 4D, compare lane 7 with lane 6). Taken together, the data from the two double digests is consistent with the predicted structure of the DHJS, as well as the predicted location of the psoralen cross-links.

Resolution of the DHJS by T7 Endonuclease I—We next tested the structure of the substrate using the HI resolvase, T7 endonuclease I, to specifically cleave at the HIIs. This enzyme has been well characterized and robustly cleaves HIIs, DNA mismatches, and to a lesser degree nicked DNA (28). Fig. 5A shows the predicted products of the digestion of the DHJS with T7 endo I. As in the model of Szostak et al. (3), the dHI can be cleaved by the resolvase in two different ways, parallel (two DNA strands are cut twice; Fig. 5A, V + V and H + H) or perpendicular (all four DNA strands are cut once; Fig. 5A, V + H and H + V). If the substrate is digested in a perpendicular fashion, analogous to gene conversion with exchange of flanking sequences, then the resulting molecule would be a large circle containing two BamHI sites, one from sequence A and another from sequence B (Fig. 5A, molecule I). Although this large circle contains the same number of base pairs as the DHJS, it would be expected to migrate slower during electrophoresis because of its more extended structure. If the substrate is resolved by the parallel pathway, analogous to gene conversion without exchange of flanking sequences, then the products of the reaction would be two small circles, A and B (Fig. 5A, molecules II and III).

Upon incubation of the DHJS with T7 endo I, products are generated that are consistent with these predictions. T7 endo I creates a pair of faster migrating bands, relative to the unreacted substrate, which migrate with similar mobilities as the undigested markers A and B, as well as a slower migrating band, which is consistent with the large dimer circle (Fig. 5B, lanes 1–4). There are other bands present in Fig. 5B, lane 2 that are consistent with the linearization of the resolved circles, which is likely a result of secondary cleavage of the DNA at a nick by T7 endo I (Fig. 5C). The identities of the electrophoretically retarded bands generated by T7 endo I can be confirmed by an additional digestion by the restriction enzyme BamHI. When the T7 endo I products are digested by BamHI, they collapse down to two bands which co-migrate with the digested markers A and B (Fig. 5B, lanes 6–9), which is consistent with the model presented in Fig. 5A.

We decided to exploit the ability of T7 endo I to cleave opposite the nick left behind from its initial resolvase cut to help us map the locations of the HIIs (Fig. 5D). The DHJS was incubated with an excess of T7 endo I, and the reaction products separated on a 15% polyacrylamide gel (Fig. 5E). There are five distinct bands that result from this reaction. Upon analysis of the gel, the three smallest bands are calculated to be 170, 253, and 302 bp in size. They correlate well with linearized fragments from...
the homologous and non-homologous sequence between the HJs, with predicted sizes of 165, 251, and 300 bp.

The sizes of the two larger bands, ~416 and ~465 bp, correspond to linearized A and B circles. One possible mechanism to generate them is that the initial cleavage at the HJ relieves the topological constraints, thus allowing the branch migration of the other HJ across the homologous region. Following the conversion of single strand nicks to double stranded breaks by T7 endo I, linearized A and B circles could then be produced.

Taken together, the restriction enzyme and T7 endo I analysis is entirely consistent with the DHJS containing two DNA circles connected by two HJs. The restriction enzyme analysis showed that the HJs are free to migrate once the topological constraint is relieved. In addition, the complete digestion of the substrate by T7 endo I also indicates that the HJs are situated at the refractory boundaries created at the transition from the homologous to the non-homologous sequences in at least the majority of the molecules.

Concluding Remarks—The DHJS is most easily thought of as two DNA circles connected by two HJs. The HJs are separated by 165 bp of homologous sequence. This allows for the HJs to be mobile; however,
the HJs cannot spontaneously migrate toward or away from each other because of the topology of a dHJ. If one views the substrate as two DNA circles conjoined by a dHJ, then the linking number between the two circles is a function of the distance between the HJs. Because the linking number cannot change without nicking or breaking the DNA, the distance between the two HJs cannot spontaneously change without inducing writhe within the substrate. Therefore, after synthesis, the two HJs are located near the refractory boundaries created by the borders of the homologous and non-homologous regions and remain there until acted upon by an enzyme that can relieve the topological constraint on the structure, such as an endonuclease or topoisomerase. Because of the distance between the HJs in this substrate (165 bp), the conjoined circles possess a linking number of \( -30 \), providing a more rigorous test for enzymes that are believed to resolve this structure through collapse of the dHJ. This substrate, and derivatives of it, should prove useful for the analysis of protein complexes that are thought to process dHJs, whether they do so by a resolvase mechanism or through convergent migration of the HJs.

Acknowledgments—We are grateful to Drs. Chapin Rodriguez and Paul Sadowski for a kind gift of the expression vector of reverse gyrase and Cre recombinase, respectively. We also thank Carrie Reardon for excellent technical assistance and lab members and our colleagues for stimulating discussions.

REFERENCES
1. Paques, F., and Haber, J. E. (1999) Microbiol. Mol. Biol. Rev. 63, 349 – 404
2. Resnick, M. A. (1976) J. Theor. Biol. 59, 97 – 106
3. Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J., and Stahl, F. W. (1983) Cell 33, 25 – 35
4. Alani, E., Padmore, R., and Kleckner, N. (1990) Cell 61, 419 – 436
5. Bishop, D. K., Park, D., Xu, L., and Kleckner, N. (1992) Cell 69, 439 – 456
6. Sun, H., Treco, D., and Szostak, J. W. (1991) Cell 64, 1155 – 1161
7. Collins, I., and Newlon, C. S. (1994) Cell 76, 65 – 75
8. Schwacha, A., and Kleckner, N. (1994) Cell 76, 51 – 63
9. Schwacha, A., and Kleckner, N. (1995) Cell 83, 783 – 791
10. Hunter, N., and Kleckner, N. (2001) Cell 105, 59 – 70
11. Heyer, W. D. (2004) Curr. Biol. 14, R56 – 58
12. Heyer, W. D., Ehmsen, K. T., and Solinger, J. A. (2003) Trends Biochem. Sci. 28, 548 – 557
13. Symington, L. S., and Holloman, W. K. (2004) Science 303, 184 – 185
14. Borner, G. V., Kleckner, N., and Hunter, N. (2004) Cell 117, 29 – 45
15. Guillon, H., Baudat, F., Grey, C., Liskay, R. M., and de Massy, B. (2005) Mol. Cell 20, 563 – 573
16. Nasmyth, K. A. (1982) Annu. Rev. Genet. 16, 439 – 500
17. Wang, J. C. (2002) Nat. Rev. Mol. Cell. Biol. 3, 430 – 440
18. Fu, T. J., Tse-Dinh, Y. C., and Seeman, N. C. (1994) J. Mol. Biol. 236, 91 – 105
19. Wu, L., and Hickson, I. D. (2003) Nature 426, 870 – 874
20. Blanton, H. L., Radford, S. J., McMahan, S., Kearney, H. M., Ibrahim, J. G., and Sekelsky, J. (2005) PLoS Genet. 1, e40
21. Hilliker, A. J., Harauz, G., Reaume, A. G., Gray, M., Clark, S. H., and Chovnick, A. (1994) Genetics 137, 1019 – 1026
22. Rodriguez, A. C., and Stock, D. (2002) EMBO J. 21, 418 – 426
23. Shatkay, A. C., and Sadowoski, P. D. (1997) J. Biol. Chem. 272, 5695 – 5702
24. Plank, J. L., Chu, S. H., Pohlhaus, J. R., Wilson-Sali, T., and Hsieh, T. S. (2005) J. Biol. Chem. 280, 3564 – 3573
25. Breguex, D., and Doetsch, P. W. (2004) BioTechniques 37, 760 – 762, 764, 766
26. Hearst, J. E. (1981) Annu. Rev. Biophys. Bioeng. 10, 69 – 86
27. Zhen, W. P., Buchardt, O., Nielsen, H., and Nielsen, P. E. (1986) Biochemistry 25, 6598 – 6603
28. Dickie, P., McFadden, G., and Morgan, A. R. (1987) J. Biol. Chem. 262, 14826 – 14836