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Padlock oligonucleotides for duplex DNA based on sequence-specific triple helix formation

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ABSTRACT **An oligonucleotide was circularized around double-stranded DNA thanks to triple helix formation. Short oligonucleotides are known to be able to form DNA triple helices by binding into the DNA major groove at an oligopurine·oligopyrimidine sequence. After sequence-specific recognition of a double-stranded DNA target through triple helix formation, the ends of the triplex-forming oligonucleotide were joined through the action of T4 DNA ligase, thus creating a circular DNA molecule catenated to the plasmid containing the target sequence. The labeling of the double-stranded DNA sequence has been carried out without any chemical or enzymatic modification of this sequence. These "padlock" oligonucleotides provide a tool to attach a noncovalent tag in an irreversible way to supercoiled plasmid or other double-stranded DNAs. Such a complex may find applications in the development of new techniques for duplex DNA detection or plasmid delivery methods for gene therapy.**

Synthetic oligonucleotides have proved to be useful tools for molecular genetic analysis, thanks to their ability to hybridize to complementary single-stranded DNA or RNA sequences. Short oligonucleotides can also bind specifically to double-helical DNA to form a local triple helix structure (1, 2), and more than a decade of research has provided a wealth of information on the parameters that govern their structure and stability (3). Triple helix-forming oligonucleotides bind to the major groove of double-stranded DNA by establishing noncovalent interactions. Although triple helix-forming oligonucleotides have been developed with the hope of artificially controlling the expression of specific genes at the level of transcription, they can also be used for other approaches. For example, triple helix formation has been used for the purification of plasmids (4, 5). Triplex-forming oligonucleotides have also been chemically modified to induce irreversible modifications of their nucleic acid target. Cleavage of the target can be achieved with an oligonucleotide covalently linked to a Fe–EDTA (6) or a Cu–phenanthroline (7) complex, whereas psoralen-oligonucleotide conjugates can be crosslinked to their target after UV irradiation (8).

In recent years, circular oligonucleotides have been the focus of some interest because of their unusual molecular recognition properties (9). They offer some improvement compared with linear oligonucleotides as tools for genetic analysis (10, 11), especially as they can be efficiently amplified by rolling-circle replication (12–15). Binding of a preformed circular oligonucleotide to double-stranded DNA can occur in two topologically distinct ways, one in which the oligonucleotide winds around the DNA and binds it from the major groove, and another one in which the oligonucleotide encircles the double helix, provided a short DNA fragment with *free ends* is used so that the circle can enter from one end around the duplex structure (16).

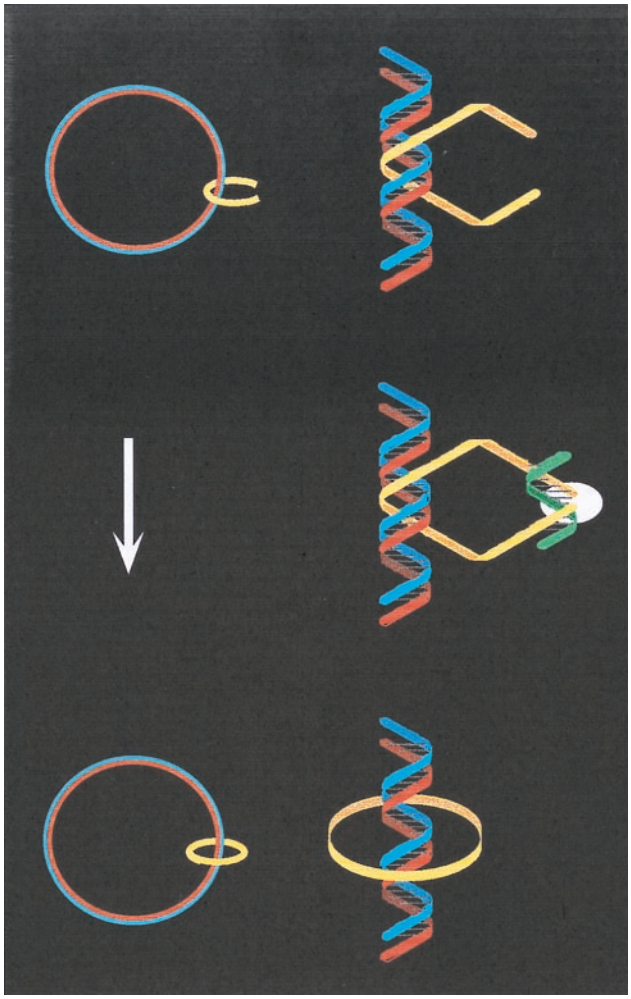

FIG. 1. Schematic diagram of the locking technique described in this paper. The central part of a linear oligonucleotide (yellow) binds to the major groove of a specific sequence within a plasmid (red and blue) to form a triple-helical complex. Then, its 5′ and 3′ ends hybridize adjacent to each other to a template oligonucleotide (green) and are joined together by T4 DNA ligase (white) to form a circular oligonucleotide catenated to the plasmid DNA.

Here, we describe an enzymatic method that allows one to assemble an artificial sliding clamp directly on a circular plasmidic DNA without any enzymatic or chemical modification of the target double-stranded DNA, through the use of a triple-helical complex intermediate (Fig. 1).

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MATERIALS AND METHODS

DNA Oligonucleotides and Enzymes. All oligonucleotides were purchased from Eurogentec (Seraing, Belgium). Concentration was measured by UV spectroscopy. Restriction enzymes (*Bam*HI, *Bsi*WI, and *Xho*I), T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. For radioactive labeling, the 89-mer oligonucleotide (10 pmol) was incubated in 20 μl of T4 polynucleotide buffer (New England Biolabs) in the presence of [γ-^{32}P]ATP (Amersham). Unincorporated ATP was removed by centrifugation in Microspin columns (Millipore).

Triplex Formation and Oligonucleotide Circularization. For triple helix formation, the labeled 89-mer oligonucleotide (100 fmol) was incubated in 10 μl of 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/10 mM DTT, 1 mM ATP/25 μg/ml BSA, in the presence of various amounts of double-stranded target. The samples were heated to 75°C, then cooled slowly to 45°C. Ligation reactions were carried out in the same buffer, by adding the template oligonucleotide (1 pmol) and 40 units of T4 DNA ligase, and incubating for 1 hr at 45°C. Ligase was heat inactivated for 15 min at 65°C. In some samples, restriction endonucleases were added directly in this mixture.

Sample Analysis by Gel Electrophoresis. Before loading on a nondenaturing gel for electrophoresis, samples were mixed with 40% sucrose to adjust the final concentration to 10%. The 6% polyacrylamide gel was prepared with a 29:1 acrylamide to bisacrylamide ratio. The gel and buffer contained TBE (100 mM Tris/90 mM boric acid/1 mM EDTA, pH 8.3) or TBE plus 10 mM $MgCl_2$, as indicated, and the electrophoresis was performed at 37°C. For denaturing electrophoresis, samples were precipitated with ethanol and resuspended in an 80% formamide/10 mM NaOH/1 mM EDTA loading buffer, heated to 90°C for 5 min, then loaded on a denaturing 6% polyacrylamide gel containing 8 M urea.

RESULTS

Triple Helix Formation. Our purpose was to design an oligonucleotide that could wind around the DNA double helix and then be circularized. The promoter of the mouse androgen receptor (17) contains a very long oligopurine·oligopyrimidine sequence which can be targeted by triplex forming oligonucleotides. *In vitro* experiments have shown that the oligonucleotide 5′-GAGGGGAGGGGAGGG-3′ can form a stable triple helix on this target (C.E. and P. B. Arimondo, unpublished results). On the basis of this sequence, we constructed an 89-mer oligonucleotide that contained a central triple helix-forming sequence connected by two T_{19} linkers to sequences that could form 10 base pairs each with a 20-mer oligonucleotide (Fig. 2). The total length of the oligonucleotide was expected to allow for binding to both the duplex target by forming a 15-base-triplet triple helix and to a 20-mer template by forming a 20-bp double helix. A phosphate group was added to the 5′ end of this oligonucleotide, as required for enzymatic circularization. [γ-^{32}P]ATP was used so that the oligonucleotide was radiolabeled.

Triple helix formation between the 89-mer and its target sequence on a plasmid was checked by using a comigration assay (Fig. 3). Staining of the gel with ethidium bromide (not shown) showed that the supercoiled plasmid barely enters into the gel (lane 2), and comparison with the electrophoretic mobility of molecular size markers indicated that the band in lane 3 migrates like a 700-bp duplex. When the oligonucleotide and plasmid pAR1 were incubated together, the labeled oligonucleotide comigrated with the plasmid (lane 2). This did not happen with the pAR4 plasmid (lane 4), in which triple helix formation was abolished by six G-to-T mutations in the target sequence (see Fig. 2). When pAR1 had been digested by both *Xho*I and *Bam*HI (lane 3), the labeled oligonucleotide migrated as a 700-bp fragment, which was expected if this oligonucleotide comigrated with the 678-bp *Bam*HI–*Xho*I fragment of pAR1 containing the target sequence for triplex formation.

Circularization Experiments. We then checked whether the 89-mer could be circularized by using T4 DNA ligase in the presence of the 20-mer template. Ligation products could be separated by denaturing gel electrophoresis. In the presence of the 20-mer template, the linear 89-mer (Fig. 4, lane 1) was very

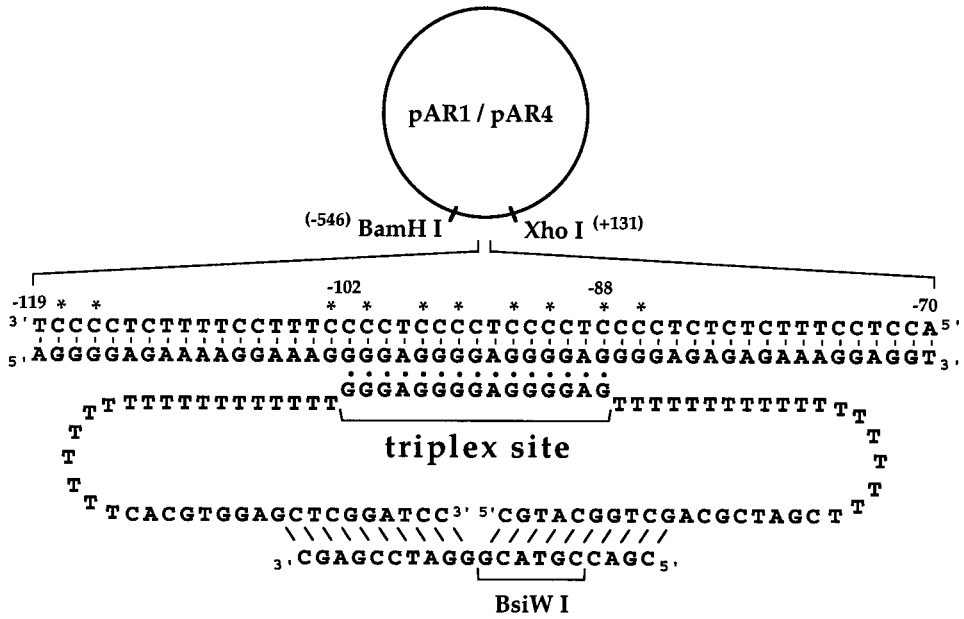

FIG. 2. Description of the plasmids and oligonucleotides used in this study. Plasmid pAR1 was constructed by insertion of the androgen receptor promoter (−546, +131) between the *Bam*HI and *Xho*I sites of the pBL-CAT plasmid (17). The sequence corresponding to positions −119 to −70 is indicated. The target sequence for triple helix formation is located between positions −102 and −88 on the promoter sequence. Sequences of the 89-mer oligonucleotide and the 20-mer template are shown. The 89-mer can form a triple helix by binding to a 15-bp sequence target in the pAR1 plasmid. In the pAR4 plasmid, the G·C base pairs indicated by an asterisk were mutated to T·A base pairs. Position of the restriction site for *Bsi*WI within the 20-bp double helix is also indicated.

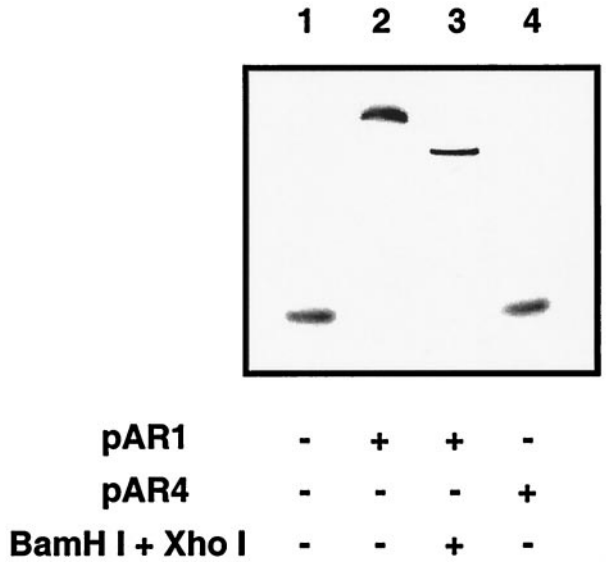

FIG. 3. Analysis of triple helix formation by a comigration assay. The labeled 89-mer oligonucleotide was incubated in 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/10 mM DTT/1 mM ATP/25 μg/ml BSA, in the absence of any plasmid (lane 1) or in the presence of 60 fmol of supercoiled pAR1 (lane 2), 60 fmol of pAR1 digested by *Bam*HI and *Xho*I (lane 3), or 60 fmol of pAR4 (lane 4). Samples were loaded on a 6% polyacrylamide gel and migrated at 37°C in TBE buffer containing 10 mM $MgCl_2$.

efficiently converted to a circular molecule, with a slower migration in a 6% denaturing polyacrylamide gel (Fig. 4, lane 2). Ligation was carried out at 45°C to reduce the formation of linear dimer molecules, appearing as a consequence of ligation of two different 89-mer oligonucleotides hybridizing to the 20-mer template. These dimers could be detected as a faint band on overexposed gels (not shown).

Ligation was also performed in the presence of the pAR1 plasmid, under conditions where triple helix is formed. The labeled oligonucleotide was converted to a species with a very slow migration rate (Fig. 4, lane 4). Migration of this species in a 1% agarose gel showed that the radiolabeled oligonucleotide comigrates with the supercoiled plasmid, as revealed by ethidium bromide staining and autoradiography (not shown).

Characterization of the Complex. We probed the nature of this species in two different ways. Firstly, the sample was treated with the restriction endonuclease *Xho*I, which linearizes the plasmid pAR1. This treatment released the circular oligonucleotide as shown by the electrophoretic mobility (Fig. 4, lane 6). Secondly, the sample was treated with the restriction endonuclease *Bsi*WI. This enzyme does not cleave pAR1, but can cleave the circular oligonucleotide in the presence of the 20-mer template. The cleavage product migrated exactly as the linear 89-mer oligonucleotide (Fig. 4, lane 5). The slowly migrating species could not be detected when pAR4, which does not form a triplex with the 89-mer, was used instead of pAR1 (lane 8), when the plasmid pAR1 was added after circularization of the 89-mer and heat denaturation of T4 DNA ligase (lane 7), and also when another linear oligonucleotide that cannot form a triple helix was used for circularization (results not shown).

These experiments proved that the oligonucleotide had been circularized around the double-stranded DNA target and therefore was catenated to the plasmid. The two molecules remained associated under highly denaturing conditions and could be separated only by breaking chemical bonds.

Diffusion of the Circular Oligonucleotide Under Triplex-Denaturing Conditions. In this complex, the circular oligonucleotide, which can form a triple helix, may be free to diffuse

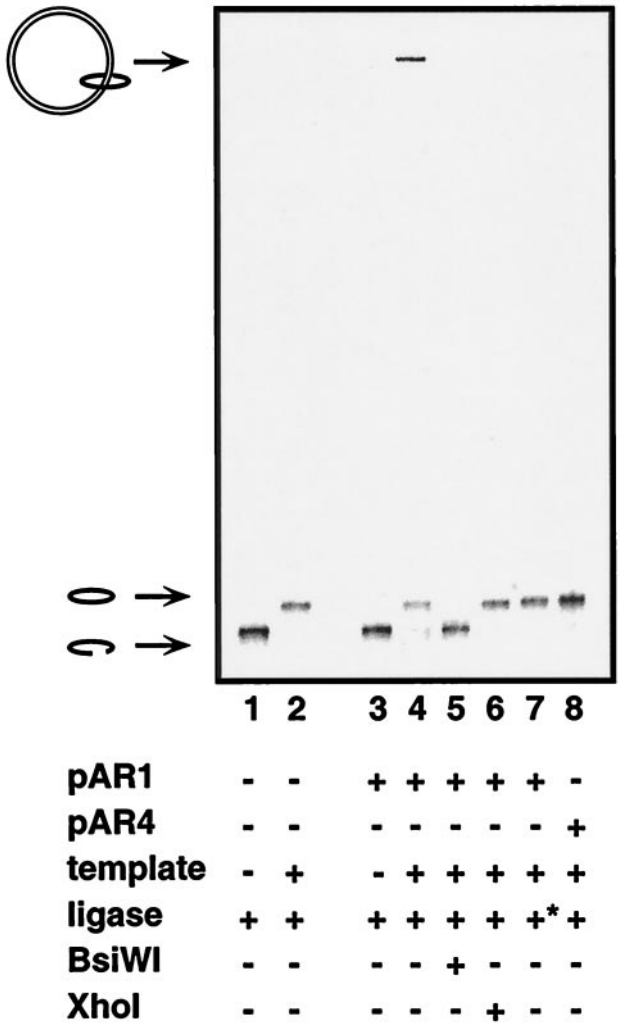

FIG. 4. Analysis of ligation and cleavage products by *denaturing* gel electrophoresis. The labeled 89-mer oligonucleotide was incubated in the absence or presence of plasmids, then treated as described below the gel. In lanes 3–6 and 8, the plasmid was present during the ligation reaction. In lane 7, the plasmid was added after circularization of the 89-mer, followed by heat denaturation of DNA ligase, as indicated by the *. Samples were analyzed on a 6% denaturing polyacrylamide gel.

in one dimension along the double helix, like a ring on a string. Triple helices with a purine-rich third strand require the presence of magnesium or any other divalent cation (18). Triple helix formation between the linear 89-mer and the pAR1 plasmid was not detected when samples incubated in a buffer containing magnesium migrated in a magnesium-free nondenaturing gel at 37°C in TBE buffer (Fig. 5, lane 2). The radiolabeled oligonucleotide remained associated with the plasmid only when it had been circularized in the presence of plasmid and magnesium before gel loading (lane 4). When the plasmid was digested by *Xho*I, the circular oligonucleotide remained associated with the linear plasmid when the gel contained magnesium (not shown), but was dissociated from the linear plasmid in the absence of magnesium, even though the cleavage site was located more than 200 bp away from the triple helix target site. This observation suggests that removing magnesium was sufficient to disrupt triple helix formation and thus allowed the circular oligonucleotide to move around the plasmid. Other strategies may be employed to label the plasmid in such a way that the triple helix interaction will be lost after ligation has occurred—e.g., ligand-induced triple helix formation (19) or pH-induced triple helix formation (3).

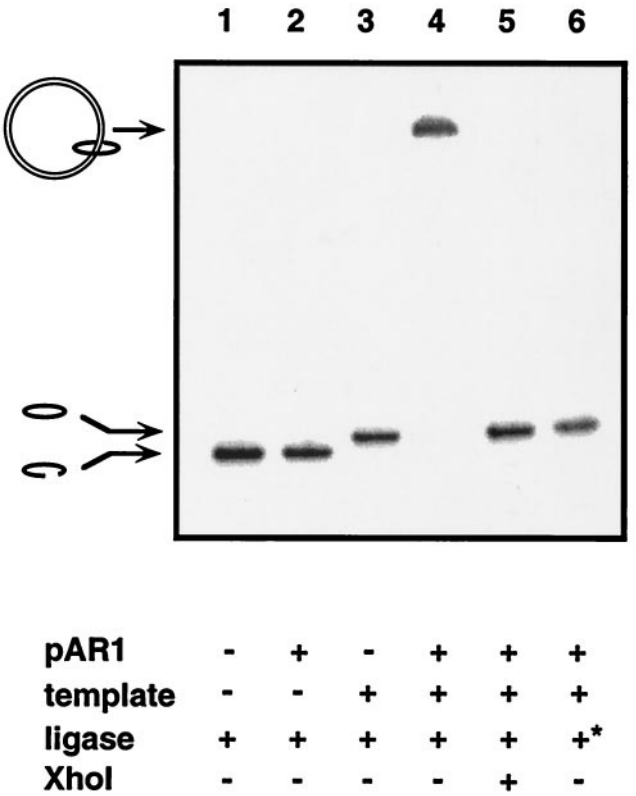

FIG. 5. Dissociation of triple helices in the absence of magnesium in a nondenaturing gel. Samples were prepared in buffer containing 10 mM $MgCl_2$, loaded onto on a magnesium-free 6% polyacrylamide gel, and then made to migrate at 37°C in TBE buffer in the absence of $MgCl_2$. Under these triplex denaturing conditions, only the circular padlock comigrates with the plasmid. Plasmid cleavage by *Xho*I allows the circular padlock to slide away from the linear duplex. In lane 6, the plasmid was added after circularization of the 89-mer, followed by heat denaturation of DNA ligase.

DISCUSSION

The method described in this paper allows one to attach a "padlock oligonucleotide" to a plasmid, provided that this plasmid contains an appropriate target sequence for triple helix formation. Attachment of this tag does not require any modification of the double-stranded DNA itself, and the tag may be free to move around the plasmid under conditions where the triple helix is dissociated. This padlock oligonucleotide can therefore constitute a tag for supercoiled plasmids.

In this study, the oligonucleotide was radioactively labeled, but other types of labeling, for example fluorescent tags, could be used. Alternatively, the circular oligonucleotide may be used for hybridization to another labeled probe or detected by rolling circle amplification (12–15). This procedure was shown to allow visualization of individual circular molecules. Therefore, the application of this method to the encircled plasmids described in this paper may allow us to count the plasmids within a cell, whereas fluorescence *in situ* hybridization (FISH)-based methods allow the measurement of transfection efficiency only when the plasmid is present in a very high copy number (20).

These padlock oligonucleotides could be linked to various chemical moieties. For example, biotin or digoxigenin may be used for capture procedures, which could lead to improved plasmid purification protocols (4, 5). Recently, different groups have attached peptides corresponding to the simian virus 40 large T antigen nuclear localization signal to plasmids (21–23). Covalent modifications of the plasmid generally interfere with reporter gene expression (21, 22), but this work has emphasized the need for a new DNA vector chemistry to enhance the efficiency of cellular gene transfer (21). Our padlock oligonucleotides provide such a noncovalent DNA vector chemistry and may therefore find applications in intracellular delivery and targeting of plasmids for applications in gene therapy.

Although triple helix formation is mostly restricted to oligopurine·oligopyrimidine sequences, these target sequences occur very commonly within genomic DNA, and there has been some improvement in the range of sequences that can now be recognized through triplex formation (3). The circularization reaction could thus be performed *in situ* on immobilized genomic DNA from cytological samples, to form a catenated complex with a natural double-stranded target, as so-called "padlock probes" do for single-stranded nucleic acids (10, 11). Formation of a complex with double-stranded DNA would avoid the requirement for denaturing DNA to obtain single-stranded sequences. *In situ* circularization performed between two sites where DNA is bound by proteins should give a rotaxane compound, as long as the proteins remain bound to the DNA in a dumbbell-like structure. This property could be used to probe the structure of DNA and its complexes with proteins or the nuclear matrix in chromatin.

CONCLUSION

Circular oligonucleotides are known to be relatively stable within cells. Further work is needed to investigate whether the oligonucleotide circle remains bound to the plasmid within cells and what are its influences on the biological properties of the plasmid, namely its ability to replicate and be transcribed. These results also open strategies in the field of supramolecular chemistry. Macromolecular assembly can be achieved through sequence-specific recognition thanks to base triplet complementarity, and the helicity of DNA allows molecules to wind around each other. Then, chemical or enzymatic ligation methods allow the efficient circularization of oligonucleotides from linear precursors. Nucleic acids therefore provide an interesting framework for the assembly of catenane structures.

Note Added in Proof. Our attention was recently drawn to a paper by Kuhn *et al.* (24), who have described a new supramolecular structure in which a circular oligonucleotide is attached to a plasmid in a sequence-specific way. In this structure, complementary strands of double-stranded DNA are locally dissociated by using a pair of peptide nucleic acid (PNA) openers binding to one strand, and an oligonucleotide is then circularized around the other strand. This circular oligonucleotide is not able to move along the double helix.

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1. Le Doan, T., Perrouault, L., Praseuth, D., Habhoub, N., Decout, J.-L., Thuong, N. T., Lhomme, J. & Hélène, C. (1987) *Nucleic Acids Res.* **15,** 7749–7760.
2. Moser, H. E. & Dervan, P. B. (1987) *Science* **238,** 645–650.
3. Sun, J. S., Garestier, T. & Hélène, C. (1996) *Curr. Opin. Struct. Biol.* **6,** 327–333.
4. Wils, P., Escriou, V., Warnery, A., Lacroix, F., Lagneaux, D., Ollivier, M., Crouzet, J., Mayaux, J. F. & Scherman, D. (1997) *Gene Ther.* **4,** 323–330.
5. Schluep, T. & Cooney, C. L. (1998) *Nucleic Acids Res.* **26,** 4524–4528.
6. Strobel, S. A. & Dervan, P. B. (1990) *Science* **249,** 72–75.
7. François, J. C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N. T. & Hélène, C. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9702–9706.
8. Giovannangeli, C., Diviacco, S., Labrousse, V., Gryaznov, S., Charneau, P. & Helene, C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 79–84.
9. Kool, E. T. (1998) *Acc. Chem. Res.* **31,** 502–510.

10. Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B. P. & Landegren, U. (1994) *Science* **265,** 2085–2088.
11. Nilsson, M., Krejci, K., Koch, J., Kwiatkowski, M., Gustavsson, P. & Landegren, U. (1997) *Nat. Genet.* **16,** 252–255.
12. Fire, A. & Xu, S.-Q. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 4641–4645.
13. Liu, D., Daubendiek, S. L., Zillman, M. A., Ryan, K. & Kool, E. T. (1996) *J. Am. Chem. Soc.* **118,** 1587–1594.
14. Lizardi, P. M., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D. C. & Ward, D. C. (1998) *Nat. Genet.* **19,** 225–232.
15. Baner, J., Nilsson, M., Mendel-Hartvig, M. & Landegren, U. (1998) *Nucleic Acids Res.* **26,** 5073–5078.
16. Ryan, K. & Kool, E. T. (1998) *Chem. Biol.* **5,** 59–65.
17. Kumar, M. V., Jones, E. A., Grossmann, M. E., Blexrud, M. D. & Tindall, D. J. (1994) *Nucleic Acids Res.* **22,** 3693–3698.
18. Malkov, V. A., Voloshin, O. N., Soyfer, V. N. & Frankkamenetskii, M. D. (1993) *Nucleic Acids Res.* **21,** 585–591.
19. Escudé, C., Sun, J. S., Nguyen, C. H., Bisagni, E., Garestier, T. & Hélène, C. (1996) *Biochemistry* **35,** 5735–5740.
20. Cheng, L., Bucana, C. D. & Wei, Q. (1996) *BioTechniques* **21,** 486–491.
21. Sebestyen, M. G., Ludtke, J. J., Bassik, M. C., Zhang, G., Budker, V., Lukhtanov, E. A., Hagstrom, J. E. & Wolff, J. A. (1998) *Nat. Biotechnol.* **16,** 80–85.
22. Ciolina, C., Byk, G., Blanche, F., Thuillier, V., Scherman, D. & Wils, P. (1999) *Bioconjug. Chem.* **10,** 49–55.
23. Zanta, M. A., Belguise-Valladier, P. & Behr, J. P. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 91–96.
24. Kuhn, H., Demidov, V. V. & Frank-Kamenetskii, M. D. (1999) *Angew. Chem. Int. Ed. Engl.* **38,** 1446–1449.