[Frontiers in Bioscience 13, 737-744, January 1, 2008]

Targeted gene modification by oligonucleotides and small DNA fragments in eukaryotes

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1. ABSTRACT

Targeted modification of genomic DNA (gDNA) in a cell permanently changes its phenotype. Numerous methods to modify target portions of gDNA in eukaryotes have been developed, and several have found practical application (e.g., homologous recombination-utilized modification in mouse ES cells). The past decadehas seen active research both in oligonucleotide-directed modifications (where the oligonucleotides can be chimeric RNA/DNA oligonucleotides, single-stranded oligonucleotides, or triple helix-forming oligonucleotides), and in small DNA fragment-directed targeted modifications. These modifications can alter gDNA at several basepairs. In order to specifically direct the reaction to the targeted site, short oligonucleotides (shorter than 80-mers) or small DNA fragments (500~2000 bps) are needed. Such oligonucleotides and small DNA fragments can be prepared easily. Although the mechanism by which targeted modification occurs has not been fully elucidated, numerous eukaryotic target genes have been modified using these methods in both cultured cells and live animals. The effectiveness of each method has been confirmed, but the efficiency of modification for all these approaches is presently insufficient for practical use. Further studies, including elucidation of complete mechanisms and optimization of methods for introducing oligonucleotides or small DNA fragment into cell nuclei, are required.

2. INTRODUCTION

Artificial modification of genomic DNA (gDNA) is one of the most effective ways to change the phenotype of a cell. Once the desired modification is achieved in a cell, the modification will be propagated in succeeding generations, therefore maintaining the phenotype permanently in the cell population. There are numerous methods for modifying targeted portions of gDNA in eukaryotes. One of the most popular methods is gene targeting of mouse ES cells by homologous recombination. In order to destroy the function of the targeted gene (knockout), a homologous sequence with a selectable marker gene in medio can replace the target sequence in a gene by homologous recombination in ES cells, thus destroying the gene's function. A similar strategy has been used to integrate a desired sequence into a targeted portion of the genome (knockin). Both these strategies can now be combined using reproduction technology, producing gene-modified mice which are essential for research into gene function *in vivo*. Other targeting methods, including the cre-loxP system (1) and adeno-associated viral vector system (2), have been developed and improved in order to achieve targeted modifications in eukaryotes.

The past decade has seen considerable research into targeted oligonucleotide-directed and small DNA fragment-directed modifications. The oligonucleotides

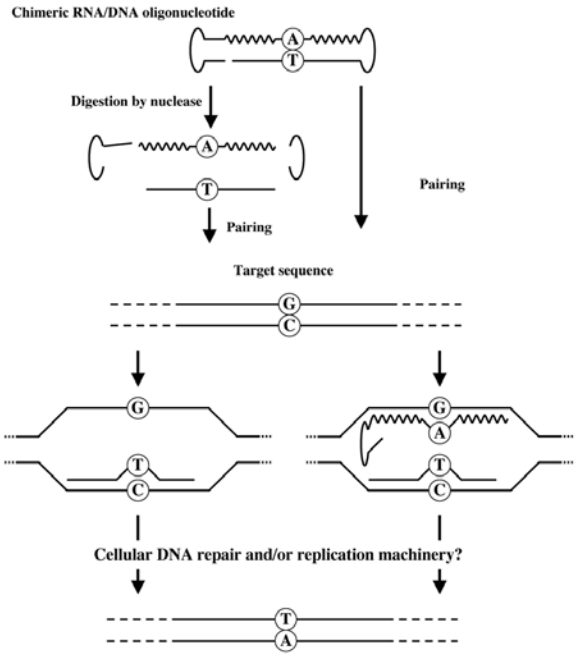

Figure 1. Chimeric RNA/DNA oligonucleotide (RDO)-directed targeted modification. Chimeric RDOs have a double hairpin at both ends, and 2'-O-methyl RNA residues; these modifications provide protection from digestion by nucleases, RNases, and hilicases. In this figure, G/C is converted to T/A by chimeric RDO. The targeted modification reaction starts with pairing whole Chimeric RDO, or single-stranded DNA released from chimeric RDO, with the target sequence. Cellular DNA repair and/or replication machinery is believed to participate in the targeted modification reaction. Wavy lines represent 2'-O-methyl RNA strands.

described in this paper are chimeric RNA/DNA oligonucleotides (RDOs), single-stranded oligonucleotides (ssONDs) and triple helix-forming oligonucleotides (TFOs). The small DNA fragments are either double- or single-stranded (typically 500-2000 bps); the general name for methods using such fragments is small fragment homologous replacement (SFHR). To achieve targeted gene modification in eukaryotes, oligonucleotides or small DNA fragments must be introduced into cell nuclei. The required oligonucleotides are easily synthesized, and small DNA fragments are also easily obtained by PCR and phargemid. The relative straightforwardness of these methods has attracted significant attention, and it is expected that these approaches will find practical use in various fields including biological science, agriculture and medical science. The utility of oligonucleotide-directed and small DNA fragment-directed targeted modifications has already been demonstrated. In the present review, each method is surveyed, including past results, notable features of the methodology, and recent advances.

3. CHIMERIC RNA/DNA OLIGONUCLEOTIDE (RDO)–DIRECTED MODIFICATION

Chimeric RDO is a synthesized oligonucleotide (approx. 70-mers) comprised of both DNA and 2'-*O*-methylated RNA residues that hybridize intramolecularly to form a double hairpin at both ends of the molecule (Figure 1). The 2'-*O*-methylation and the double hairpin together protect the chimeric RDO from digestion by RNases, helicases, and exonucleases (3). RDO has a single mismatched base pair (s) in the center of the molecule, removed from the portion of the sequence that is complimentary to the target site. In 1996, Yoon *et al*. first reported that introduction of chimeric RDOs into CHO cells could repair an extrachromosomal point-mutated alkaline phosphatase gene inserted into plasmid DNA (4). Since then, many researchers have demonstrated the utility of chimeric RDO in targeted gene modification. Although the mechanism of modification by chimeric RDO has not been fully elucidated, several studies have highlighted general features of the reaction; for example, the reaction starts with pairing of either a portion or the entire chimeric RDO with the target site, followed by cellular DNA repair and/or DNA replication (5).

Extrachromosomal genes, and chromosomal genes such as inactive tyrosinase, β-globin and apoE2, can be targets in cultured cells (3, 6-9). Targeted gene repair has also been confirmed *in vivo* including factor IX gene in mouse liver (10), UGTA1 gene with a frameshift mutation (11), inactive tyrosinase gene in mouse skin (12), and Mdx gene (analogue of Duchenne muscular dystrophy gene) in mouse muscle (13) and canine muscle (14).

Several species, including plants and yeast, have been studied. In plants, the acetolactate synthase gene in tobacco and the acetohydroxyacid synthase gene in maize have been corrected by chimeric RDOs (15, 16). Yeast has been a good eukaryotic model for investigating targeted gene repair by chimeric RDOs (17, 18). In addition, cell-free extracts prepared from mammalian and plant cell muclei, and mammalian mitochondria, have also been utilized as models for elucidating the mechanism of targeted gene repair by chimeric RDO (18-21). Thus, chimeric RDOs could potentially be applied to various species both *in vitro* and *in vivo*. However, a serious deficiency of this methodology is poor reproducibility. Replication of published studies, either by other groups or by the original, reporting laboratory, has been difficult (22). There are several reports in which modification by chimeric RDO could not be detected (23-26). Although the causes of the observed poor reproducibility have not been revealed, factors such as the target base (27), the GC content around the target base (25, 26), and the purity of the chimeric RDO (24, 26) have been suggested.

4. SINGLE-STRANDED OLIGONUCLEOTIDE (ssODN)-DIRECTED MODIFICATION

Single-stranded oligonucleotides (ssODN) for targeted gene modification have approx. 50~80 bases, and the nucleotide sequence is complementary to the sequence of the target strand except for the target base (s) (Figure 2). Point and frameshift mutations can be repaired by ssODN-directed modification (17). Introduction of ssODN into cell nuclei can trigger the targeted gene modification reaction. In very recent research, ssODNs have been modified,

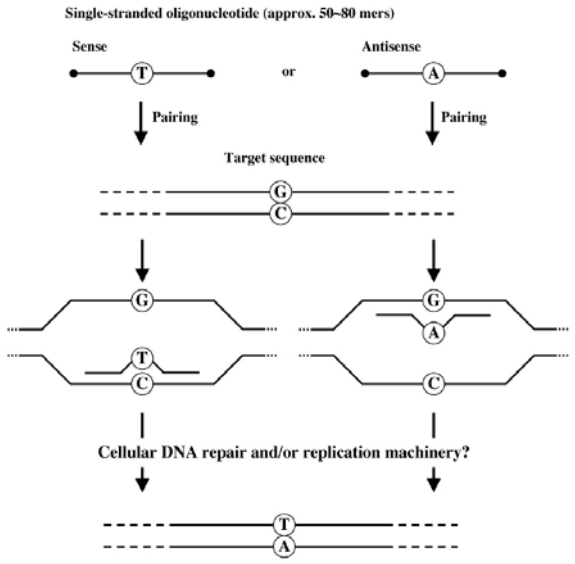

Figure 2. Single-stranded oligonucleotide (ssODN)-directed targeted modification. ssODNs typically have approx 50~80 bases, and contain 2'-O-methylated RNA residues or phosphorothioate linkages at each end (filled circles); these modifications provide protection from digestion by nucleases. Both sense and antisense oligonucleotides can be used as ssODNs. In this figure, G/C is converted to T/A by ssODN. Cellular DNA repair and/or replication machinery is believed to participate in the targeted modification reaction.

including 2'-*O*-methylated RNA residues or phosphorothioate linkages at both ends of the oligonucleotide (28, 29), in order to protect the ssODN from digestion by nucleases. Approximately 15 years ago several groups confirmed that ssODNs without any chemical modifications could induce targeted gene modification in yeast and human cells (30-33). Since then, research regarding ssODN-directed targeted gene modification has progressed, albeit slowly (34). A major breakthrough occurred in 2000 (28) when Gamper *et al.* demonstrated that ssODNs modified with phosphorothioate linikages at both ends can repair target genes more effectively *in vitro* than chimeric RDO; the effectiveness of this approach was subsequently confirmed in cultured cells (29). This finding is important because ssODNs can be synthesized more economically and simply than chimeric RDOs. In addition to increased efficiency, experimental reproducibility is higher than with the chimeric RDO-methodology (35, 36). Moreover, ssODNs are easy to work with because their structures are stable. These attributes have drawn attention to ssODN-directed targeted gene modification, and many reports have recently been published. Elucidation of the mechanisms behind the technique, and efforts to further improve modification efficiency, are currently in progress (37-40). Cellular homologous recombination, DNA replication and repair, and transcription are all believed to play important roles in the targeted gene modification reaction (34).

To date, several disease-causing genes, including the acid α-D-glucosidase, Mdx and transthyretin genes, have been repaired by ssODNs both in cultured cells and *in vivo* (41-43). Aside from gene therapy, this technique could also be useful for genetic engineering. In mouse ES cells, ssODNs can repair point-mutated reporter genes such as EGFP and LacZ in chromosomes (44, 45), suggesting that ssODN could be used to produce gene-modified mice in future. In addition, Takahashi *et al.* (2005) could repair a bruno-like gene in zebrafish embryos with relatively high efficiency (3 in 80 embryos) by injecting ssODNs and rad52 protein (which is believed to play an important role in the targeted modification reaction) into the embryos.

As described above, ssODNs have a sequence complementary to the target strand, except for the target base (s). Therefore, the ssODN sequence can be either sense or antisense. Although it has been reported that correction efficiencies using either sense or antisense ssODNs are similar (47), other reports indicate that modification efficiencies are biased depending on the exact nature of the ssODN used (strand bias) (17, 29, 42, 48). To date, several factors, including transcription activity and orientation of the target gene, GC content of the ssODN, and localization of the target gene (extrachromosomal or chromosomal) can all bring about strand bias (37, 44, 49, 50).

5. SMALL FRAGMENT HOMOLOGOUS REPLACEMENT (SFHR)

In a mammalian cell, a single- or double-stranded DNA fragment having several hundred bps (approx. 500- to 2000-mers) can replace a homologous sequence in a gene (Figure 3), permitting straightforward modification of the targeted gene. This reaction is conducted by the cell's homologous recombination machinery, so the DNA fragment need only be inserted into the cell in order to accomplish targeted gene modification. This strategy is generally termed small fragment homologous replacement (SFHR); a similar, established technique used to disrupt a targeted gene in mouse ES cells is called 'gene knockout'. Compared to this (now classic) method, SFHR has an advantage in the preparation of the DNA fragment. The gene knockout method requires that the DNA fragment have a long homologous sequence, including a selectable marker gene. Preparing such DNA fragments is difficult. In contrast, as described above, the DNA fragments used in SFHR are simple and much shorter, and so can be prepared easily. DNA fragments for SFHR are generally prepared from PCR products, but this approach can introduce unwanted mutations caused by mis-amplification by DNA polymerase. A potential solution to this problem is to use single-stranded DNA fragments prepared from phargemids (51-53): since amplification of phargemids is higher in fidelity, unwanted DNA mutations should be eliminated.

It has been demonstrated that SFHR is a potential strategy for repairing disease-causing genes. The deletion of phenylalanine 508 in the cystic fibrosis transmembrane conductance regulator gene (the most common mutation in cystic fibrosis patients) was repaired by SFHR in cultured

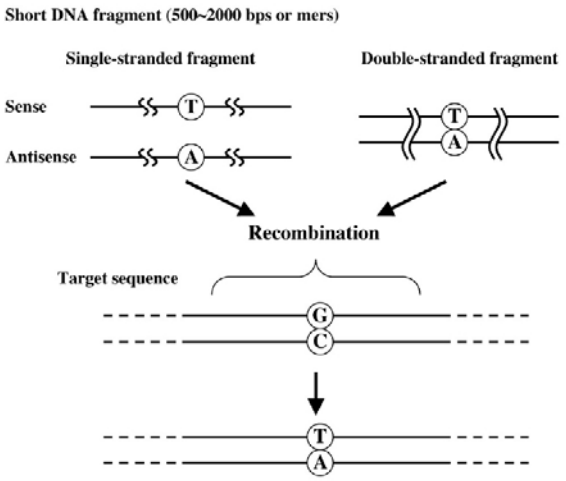

Figure 3. Small DNA fragment homologous recombination (SFHR). Small DNA fragments are double- or single-stranded DNA with a length of approx. 500~2000 bps (500- to 2000-mers. In this figure, G/C is converted to T/A by SFHR.

cells and in the lungs of live mice (54-57). Concz *et al.* (2002) altered the β^A globin gene (normal) to the β^s globin gene (which causes sickle cell anemia), suggesting that gene therapy using SFHR can be used to cure sickle cell anemia. In addition, the Mdx gene in cultured cells and in living mice (58), and a mutation in exon 7 of the SMN2 gene (which causes spinal muscular atrophy) of primary cultured cells from human placental villi (59), have both been corrected by SFHR.

As with ssODNs, strand bias has been confirmed in SFHR using single-stranded DNA fragments. Recently, sense DNA fragments complementary to the sense strand of the target region, except for a single base, were shown to modify the extrachromosomal targeted gene more efficiently (about 6-fold) than an antisense fragment (52). Strand bias in ssODN-directed modification is affected by various factors, including transcription activity, replicative direction and the location of the target gene (extrachromosomal or chromosomal) (see section "2. Single-stranded oligonucleotide-directed modification"). However, in the systems studied by Tsuchiya *et al.*, transcription activity and replication did not affect strand bias (51). Further studies will help elucidate both the mechanism behind SFHR and provide clues for making SFHR a more powerful targeted gene modification technique.

6. TIRPLE HELIX-FORMING OLIGONUCLEOTIDE (TFO)-DIRECTED MODIFICATION

Synthetic single-stranded oligonucleotides containing purines or pyrimidines (typically 10~30-mers) can bind to polypurine/polypyrimidine-rich regions of double helix DNA in a sequence-specific manner (60). Such oligonucleotides (abbreviated TFOs) have been shown to be capable of modifying genes in cultured cells and living mice.

TFO conjugated with a mutagen such as psoralen or an alkylating compound, can cause site-specific mutations in a gene (Figure 4A). For example, psoralen crosslinks bases in the vicinity of the TFO-binding site upon UVA irradiation. The crosslink is repaired by the cellular DNA repair machinery, thus completing the targeted mutation. In one study, psoralen-conjugated TFO targeted conversion of TA to AT with an efficiency of approximately 70% (61). To date, chromosomal reporter genes, including mutated versions of the supF and HPRT genes, have been corrected in cultured cells by psoralen-conjugated TFO (62, 63). Interestingly, TFOs without a conjugated mutagen can cause mutation at a TFO-binding region, although the frequency of mutation is much lower than that by mutagen-conjugated TFOs (Figure 4B) (64). The mechanism is believed to involve both transcription-coupled repair and excision repair (64). Repetitive administration of TFOs in the absence of mutagen produced specific mutations in live mice (65). In addition to mutagens, reagents that enhance site-specific delivery of TFOs can also be conjugated. For example, TFO conjugated with mannose 6-phosphate-bovine serum albumin can be introduced effectively into hepatic stellate cells of living mice (66).

Targeted gene repair has also been accomplished with TFOs using homologous recombination. A single-stranded or double-stranded DNA fragment (donor fragment) tethered covalently or via a short complementary strand to TFO can replace a homologous sequence in the targeted gene (67, 68). If the donor fragment has a sequence homologous to the target except for a specific base (or bases), the targeted sequence will be corrected after precise homologous recombination (Figure 4C). Targeted gene repair in cultured cells has been achieved using this method (69). Further studies have clarified that co-introduction of both a donor fragment and TFO can produce an identical effect (Figure 2D) in gDNA, such as the adenosine deaminase and p53 genes (70). In addition, TFO without a donor fragment can enhance intramolecular homologous recombination (Figure 4E). The TFO requires a polypurine/polypyrimidine-rich region in the vicinity of the target sequence, but since such regions occur on average every 1 kb in gDNA, most genes can be targeted using this method.

Factors affecting the affinity of TFOs have been investigated in order to make TFO methodologies more effective. For example, pyrimidine TFOs, especially C-rich sequencse, have only weak affinities for target regions in cells (71). In addition, TFO cannot bind to a target region effectively at physiological concentrations of Mg^{2+} (72). To overcome such limitations, numerous modified TFOs have been developed (72). Brunet *et al.* (2005) have reported that pyrimidine TFO, modified by locked nucleic acid and acridine, can function in cells more effectively than non-modified TFO (71). Developments relating to modified TFOs continue to be reported (73-75).

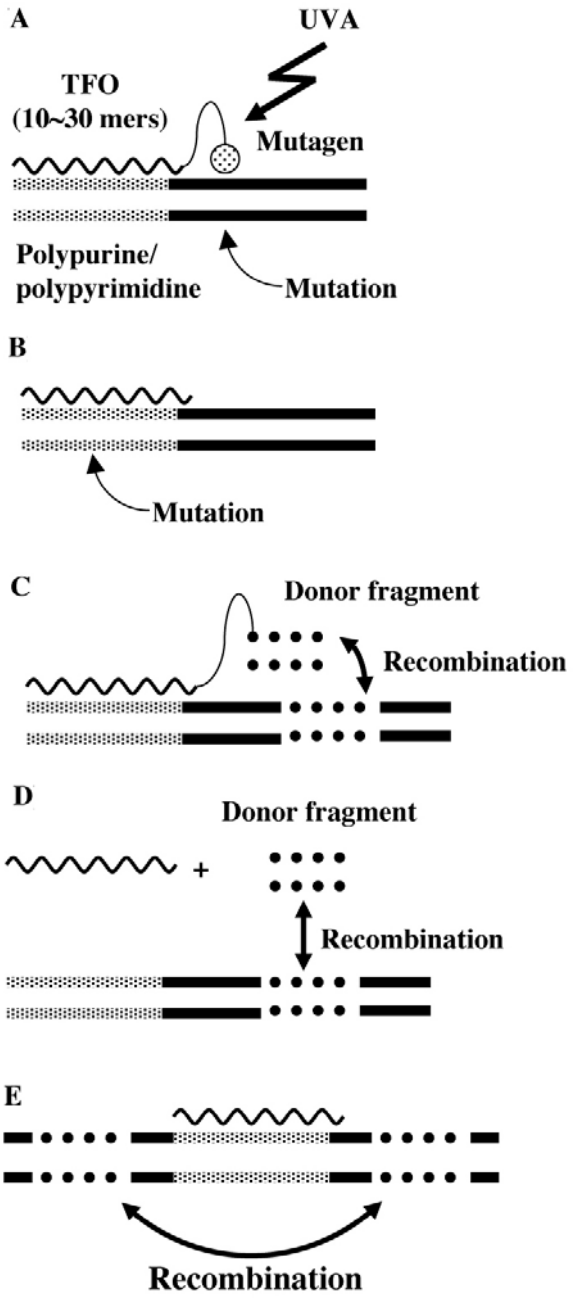

Figure 4. Triple helix-forming oligonucleotide (TFO)-directed targeted gene modification. (A) Targeted mutation.

7. PERSPECTIVES

In the present review, four methods that can modify targeted eukaryotic gDNA have been reviewed. It is clear that all four methods can modify gDNA, but to date the efficiency of modification is generally insufficient to be practical. According to data published up to 2004, efficiencies vary widely (e.g., chimeric RDO, 0.001~88%; ssODNs, 0.0001~1%; SFHR, 0.005~20%) (5). Although direct comparisons are not possible due to different materials and methods used in each study, general comparisons can nonetheless be useful when discussing modification efficiency. Presently, extremely high efficiencies are considered to be artifacts because they are estimated using PCR, an artifact-prone technique (5); therefore true efficiency using these strategies may be closer to 1%. Because of the overall low efficiency of targeted gene modification, *ex vivo* gene therapy in hematopoetic stem cells is currently the most probable application of this technique (76). If gDNA in cells obtained from a patient can be repaired, these repaired cells can in theory be selected and returned to the patient. However, in order for these methods to be practical *in vivo*, the efficiency must be significantly improved, which will require that the mechanism of targeted modification be thoroughly elucidated. If limiting factors in the mechanisms are identified, methodological steps can then be taken to improve the efficiency of the technique. Several studies have already provided some hints as to how to improve efficiency. For example, in TFO-based methods, it was discovered that the weak affinity of TFOs is a barrier to obtaining high DNA modification efficiency. Therefore, TFOs have been modified to have high affinity, resulting in improved DNA modification efficiency. Studies leading to further improvements will undoubtedly continue to be published.

Improved methodologies for introducing small DNA sequences will also continue to improve efficiency. Introducing small DNA fragments into nuclei is the first step in targeted gene modification, so without effective introduction, effective modification cannot be expected. Recently, Nakamura *et al.* (2004) reported effective targeted gene repair (approx. 10%) in mouse liver by ssODN-atelocollagen complexes. Atelocollagen protects nucleic acids from digestion by nucleases and releases the nucleic acids gradually, so the nucleic acids are introduced into cells over long periods of time. Atelocollagen has therefore recently drawn attention as a new carrier for introducing DNA in both *in vitro* and *in vivo* systems (77). Parekh-Olmed *et al.* (2005) suggested that prolonged introduction of ssODNs caused the high efficiency observed by Nakamura *et al.* (2004). It is important to confirm whether prolonged introduction is conducive to obtaining high modification efficiency. To date, several carriers possessing attributes similar to atelocollagen have been developed (e.g. cationized gelatin and chitosan) (78), and may be useful in improving targeted modification efficiency by small DNA fragments. Although this concept differs from the techniques discussed above, novel methods for introducing DNA for targeted gene modification should be explored. For example, *in vivo* electroporation (EP) can introduce DNA, RNA and protein into various organs, including muscle, testis, ovary, skin, and kidney (79). The amounts of DNA introduced by *in vivo* EP are much higher than that achieved using chemical-based methods, including lipofection. Excessive introduction of oligonucleotides into cells can cause cell death (34), but *in vivo* EP can avoid this problem because the amount of introduced DNA can be controlled easily.

In conclusion, many researchers have demonstrated targeted gene modification by the methods

described in the present review, and have confirmed their effectiveness. However, modification efficiencies are presently insufficient for practical use; further studies, including the elucidation of mechanisms and the investigation of delivery systems for oligonucleotides and small DNA fragments are required.

8. ACKNOWLEDGEMENT

I gratefully acknowledge Dr Murai (Nagoya University) for helpful discussions and suggestions.

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Key Words: RNA, DNA, Targeted, Modification, Eukaryote, Chimeric, Oligonucleotide, Single-Stranded Oligonucleotide, Small Fragment Homologous Replacement, Triple Helix-Forming, Oligonucleotide, Review

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