REVIEW

Twenty years hunting for sulfur in DNA

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ABSTRACT

Here we tell a 20-year long story. It began with an easily overlooked DNA degradation (Dnd) phenomenon during electrophoresis and eventually led to the discovery of an unprecedented DNA sulfur modification governed by five *dnd* genes. This unusual DNA modification, called phosphorothioation, is the first physiological modification identified on the DNA backbone, in which the nonbridging oxygen is replaced by sulfur in a sequence selective and stereo-specific manner. Homologous *dnd* gene clusters have been identified in diverse and distantly related bacteria and thus have drawn immediate attention of the entire microbial scientific community. Here, we summarize the progress in chemical, genetic, enzymatic, bioinformatical and analytical aspects of this novel postreplicative DNA modification. We also discuss perspectives on the physiological functions of the DNA phosphorothioate modification in bacteria and their implications.

KEYWORDS DNA sulfur modification, DNA phosphorothioate modification, DNA degradation

SITE-SPECIFIC DEGRADATION OF DNA DURING ELECTROPHORESIS

About 20 years ago, Zhou et al. noticed a DNA degradation (Dnd) phenomenon in *Streptomyces lividans* during electrophoresis—preparations of both plasmid and chromosome

DNA partially degraded on agarose gels (Zhou et al., 1988). In many of the previous publications, it was generally assumed that such degradation was caused by contamination with endogenous nucleases and the phenomenon was essentially neglected. Zhou et al. demonstrated unambiguously, however, that it was caused by an unprecedented site-specific modification in *S. lividans* in a way that renders its DNA susceptible to degradation during electrophoresis (Zhou et al., 1988).

S. lividans DNA was seen degraded during electrophoresis only in so-called “bad” Tris-acetate electrophoretic buffer. The “bad” buffer was referred to distinguish from “good” buffer, in which DNA did not degrade (Zhou et al., 1988). Close relatives to *S. lividans*, *Streptomyces coelicolor* A3(2), *Streptomyces albus* G J1074, *Streptomyces griseofuscus* 3213, and *Streptomyces ambofaciens* 3212 did not exhibit the Dnd phenomenon. Particularly, when the bifunctional plasmid pIJ699 was isolated from *S. lividans* and *E. coli*, respectively, only pIJ699 isolated from *S. lividans* was degraded under electrophoresis, indicating that the DNA was specifically modified in *S. lividans* (Zhou et al., 1988).

S. lividans DNA degraded as smears rather than sharp bands on agarose gels during electrophoresis. Linearization of degraded molecules of plasmid pIJ699 on agarose gel suggested double-stranded cleavage during electrophoresis in “bad” buffer. The average size of the chromosome DNA fragments after complete cleavage in “bad” buffer was approximately 6 kb.

The strongest evidence showing that the Dnd phenomenon was a physiological modification came from the isolation of mutant *S. lividans* strain, ZX1, obtained by NTG mutagenesis of wild-type strain JT46 (Zhou et al., 2004). In comparison to

JT46, ZX1 does not modify its DNA, and its DNA was not degraded during electrophoresis.

Moreover, *Streptomyces avermitilis* possesses an essentially identical DNA modification system to that found in *S. lividans*, as its DNA also underwent extensive degradation in gels run in Tris-containing buffer (Evans et al., 1994). DNA modification is comparable in the two species both in terms of the chemical properties of the modifications resulting in Tris-dependent cleavage and their site specificity (Evans et al., 1994).

Dyson et al. later demonstrated that this unusual modification was a postreplicative DNA modification, as the modification was not detectable in single-stranded replication intermediates (Dyson and Evans, 1998). Dyson et al. suggested that the modifying agent acted postreplicatively on unmodified double-stranded DNA substrates.

CHARACTERIZATION OF NUCLEOLYTIC SPECIES RESPONSIBLE FOR DEGRADATION IN ELECTROPHORETIC BUFFER

"Bad" buffer could be autoclaved without losing nucleolytic activity and the nucleolytic activity readily diffused through dialysis membranes, eliminating the possibility that an enzymatic activity or bacterial contamination was the cause of the degradation of *S. lividans* DNA (Zhou et al., 1988). Zhou et al. suspected that a negatively charged nucleolytic compound in the "bad" buffer might contribute to the site-specific cleavage of *S. lividans* DNA. Alternatively, ferrous ions (Fe^{2+}) and possibly molecular oxygen generated at the anode were suggested to have a role in the cleavage reaction.

Ray et al. later demonstrated that the cleavage activity was actually dependent on Tris in the electrophoretic buffer (Ray et al., 1992). When the voltage was applied, Tris buffer was activated at the anode to generate a nucleolytic species responsible for cleavage activity. A correlation between the concentration of Tris-dependent nucleolytic species and the amount of the DNA cleavage was observed in an *in vitro* cleavage assay (Ray et al., 1992). Particularly, maximal DNA cleavage was observed after a 90-minute incubation with the activated Tris buffer adjacent to the anode. The addition of 5 μM or greater thiourea, a scavenger and reducing agent, prior to activation resulted in complete inhibition of DNA cleavage. DNA did not degrade under electrophoresis in an alternative Hepes buffer.

The incubation of modified DNA with Tris buffer prior to the activation does not result in DNA cleavage. However, the addition of per-acetic acid to inactive Tris buffer led to a significant level of cleavage, implying that the Tris derivative is an oxidant. Moreover, the primary amine group of Tris was shown to be critical for DNA cleavage based on the observation that an analogue of Tris, Tris ethane, in which the amine group is replaced by a methyl group, did not have

cleavage activity after electrophoretic activation (Ray et al., 1995). In particular, the generation of oxidant could be measured using reduced methyl viologen. In addition, certain reducing agents could inhibit DNA cleavage. Hence, the Tris derivative is regarded as a bifunctional reagent containing both per-acid and primary amine groups. In accordance with this hypothesis, lysine and histidine, bifunctional reagents possessing primary amine groups and potentially per-acid derivatives oxidized at their carboxyl groups, also exhibited DNA cleavage activity after electrophoretic or chemical activation. Ray et al. thus concluded that the Dnd phenomenon in electrophoresis was due to peracid-mediated oxidative, amine-catalyzed attack at the modified DNA sites (Ray et al., 1995).

PREFERRED MODIFICATION SITES AND RECOGNITION SEQUENCES

The preferential cut site on pIJ101, a plasmid from *S. lividans*, was roughly located between *Bam*HI and *Kpn*I sites (Zhou et al., 1988). Detailed characterization of the degraded linear fragments of plasmid pIJ101 isolated from *S. lividans* revealed that the DNA could be cut at several specific sites. The chemical sequencing pattern showed a readable sequence ladder, suggesting that the DNA was cut at specific nucleotides (Zhou et al., 1988; Evans et al., 1994). Plasmid pIJ303 reisolated after introduction into *S. lividans*, and *S. avermitilis* gave similar fragment profiles. All DNA fragment products were not produced in equal proportions. Fragment sizes were calculated and a map of fourteen predominant cleavage sites was assembled (Evans et al., 1994).

To determine the precise location of preferred pIJ101 modification sites, a 160-bp fragment containing the site was cloned into the shuttle vector pUCS75 to enable modification in *S. lividans* (Dyson and Evans, 1998). Cleaved and uncleaved plasmid DNAs were employed for an *in vitro* primer extension assay to locate the cleavage sites (Dyson and Evans, 1998). The modifications were determined to occur at guanine residues on either strand within a 6-bp palindromic sequence. Furthermore, Dyson et al. revealed that the modification required the region surrounding the central 6-bp palindrome as part of the substrate. The essential region contains three 13-bp imperfect direct repeats with the 6-bp core sequence lying in the middle one, with two long inverted repeats of different sequence flanking the core-containing sequence. The left-hand and right-hand direct repeats overlap the arms of the inverted repeats. The complete deletion of either the inverted repeat or the overlapping direct repeat prevented the modification within the 6-bp core sequence (Dyson and Evans, 1998).

The recognition sequences were further investigated by examining the preferred modification sites in a 5.7-kb amplified DNA sequence (ADS_{5.7}) of a chromosomal element from specific *S. lividans* mutants (Boybek et al., 1998).

Boybek et al. employed primer extension assays and chemical sequencing to localize four modification sites in the ADS_{5.7}. Central core sequences were again detected in each of the recognition sites. Notably, a palindromic 8 nt (5'-CcGGCCgG-3') with a central 5'-GGCC-3' core sequence was highly conserved between different recognition sequences. This was also true for the core sequence from plasmid pIJ101. The modification sites were located on either, and occasionally both, of the two central guanine residues and a guanine on the opposite strand separated by a distance of 2 or 3 bp. There were no significant similarities, however, between flanking sequences surrounding the central core sequence of different modification sites. Although, like pIJ101, modification within ADS_{5.7} also required extensive sequences surrounding the core sequence, there were no significant inverted repeats in any of the sequences, no direct repeats > 6 bp in length shared between all sites, and no good matches to the 13-bp repeats of the pIJ101 site. The authors suggested that the repeat structures present in the pIJ101 site could contribute to the greater susceptibility to *in vivo* modification than that of the ADS_{5.7} site.

In a more recent sequence recognition specificity study, Liang et al. analyzed 14 modified sites by cloning and sequencing cleaved fragments from a pIJ101 derivative plasmid, pHZ209 (Liang et al., 2007). The highly conserved 4-bp central core 5'-GGCC-3' was found to lie within the region 5'-c-cGGCCgccg-3', in agreement with the conserved

modification sequence identified by Boybek et al. (Boybek et al., 1998) (Fig. 1). However, Liang et al. found that the two modified nucleotides on opposite strands are adjacent rather than separated by two bases within the consensus region. Further, site-directed mutagenesis of the consensus sequence demonstrated that the central core (5'-GGCC-3') was stringently required for modification in the wild type and a mutant strain, while the first (g) and the third (c) of the four nucleotides (gccg) flanking it on the right was only important for modification in the mutant (Liang et al., 2007).

THE GENE CLUSTER RESPONSIBLE FOR Dnd PHENOMENON

Initial effort for the cloning of the gene(s) responsible for Dnd phenomenon was frustrated by the lack of the easily detectable or assayable phenotype(s) for convenient or efficient screening, and we failed in shotgun cloning experiments using modification-deficient mutant ZX1 as host, neither by cloning into multi- nor low-copy plasmid vectors. An attempt to find a hypothetical phage that is able to infect modification-deficient ZX1 but not its progenitor(s) so as to screen for a possible phage-resistant gene that might be associated with the Dnd modification system from the ZX1 progenitor first was very exciting (Zhou et al., 1994a). A new *Streptomyces* phage ϕ HAU3 was discovered to possess the wanted phenotype (Zhou et al., 1994a, b) and was used for

Figure 1. The phosphorothioate modification occurs specifically between two dGs with R_p configuration in *S. lividans*.

(A) DNA sulfur modification occurs within a highly conserved 4 bp consensus "core" sequence, 5'-G*GCC-3'. In plasmid pIJ101, the modification sequence lies in the middle one (DR-2) of the three direct repeats (DR1-3). The two inverted repeats (IR-1 and IR-2), overlapping direct repeat sequences of DR-1 and DR-3, have the potential for the formation of stem-loop structures. (B) The chemical nature of DNA sulfur modification was subsequently revealed to be sequence-selective and stereo-specific phosphorothioation of the DNA backbone, in which the non-bridging oxygen is replaced by sulfur.

the screening of the hypothetical phage-resistance gene. A gene (ϕ HAU3^R) obtained that is identical to the ea59 of the *E. coli* phage λ , encoding a hypothetical endonuclease (Zhou et al., 1994b), however, seem to be independent of the Dnd modification system: the acquisition of HAU3^R did not restore Dnd phenomenon on ZX1; deletion of HAU3^R from ZX1 progenitor did not make DNA stable during electrophoresis; interruption of HAU3^R by Tn4560 did not change Dnd phenomenon; and another known *dnd*⁺*Streptomyces* species was known to be HAU3^S. Additionally, the sequence immediately flanking both side of the cloned ϕ HAU3^R gene was also irrelevant to the Dnd phenotype.

In comparison with the wild-type *S. lividans*, mutant ZX1 has a roughly 90 kb chromosomal deletion and apparently lacks the Dnd phenomenon. Notably, when a set of 13 ordered cosmids (Zhou et al., 2004) covering the deletion region were transformed into ZX1, cosmid 16C3 was found to confer ZX1 Dnd phenomenon, indicating that 16C3 contained genes for the specific DNA modification associated with the Dnd phenomenon (Zhou et al., 2005). The *dnd* gene cluster was 6 kb away from the right junction of the deletion region in ZX1 and 24 kb away from the ϕ HAU3 resistance gene (Zhou et al., 1994b, 2004).

The *dnd* locus responsible for DNA degradation was first localized to an 8 kb region of 16C3 and later precisely to a 6665-bp DNA fragment consisting of five open reading frames, designated *dndA*–*E* (Zhou et al., 2005; Xu et al., 2009). *dndA* and *dndB*–*E* are divergently transcribed, while *dndB*, *dndC*, *dndD* and *dndE* were shown by RT-PCR to constitute an operon (Zhou et al., 2005; Xu et al., 2009) (Fig. 2). Deletion of *dnd* locus abolish the DNA modification, and gain of the *dnd* locus by Dnd[−] or mutant strains conferred the DNA modification/Dnd phenomenon (Zhou et al., 2005).

Apart from the deletion of the entire *dnd* gene cluster,

targeted disruption on the chromosome or by a plasmid-based mutation-integration-complementation system clearly demonstrated that *dndA*, *dndC*, *dndD* (*spfD* in *P. fluorescens* Pf0-1), and *dndE* are essential for the DNA degradation (Zhou et al., 2005; Xu et al., 2009; Yao et al., 2009). Unlike other mutants, *dndB* mutants demonstrated significantly aggravated Dnd phenomenon (Liang et al., 2007; Xu et al., 2009). DndB was suggested to be a DNA topology-modifying protein, like a DNA gyrase, and thus, the efficiency and/or specificity of DNA modification at certain sites flanked by the sequences with potential to form secondary structures (Liang et al., 2007).

Characterization of the *dnd* gene cluster played a critical role in understanding Dnd and immediately facilitated deeper investigation into this unusual DNA modification. *dndA* is predicted to encode a protein of 380 amino acids homologous to IscS and NifS proteins in *E. coli*, which both generate sulfur via an L-cysteine desulfurase activity (Zhou et al., 2005). Purified DndA protein did show L-cysteine desulfurase activity, catalyzing removal of elemental sulfur from L-cysteine to produce L-alanine (You et al., 2007). Cys327 in the C-terminal region of DndA was demonstrated by site-directed mutagenesis to be the active enzymatic center (You et al., 2007). The DndC protein (498 aa residues) contains a [4Fe-4S] cluster and shows observable ATP pyrophosphatase activity, catalyzing hydrolysis of ATP to AMP and pyrophosphate (Zhou et al., 2005; You et al., 2007). Particularly, DndA can activate the reconstitution of the [4Fe-4S] iron-sulfur cluster in the DndC protein, while a mutated DndA_{Cys327Ser} lost its cysteine desulfurase activity and its ability to reactivate the apo-Fe DndC (You et al., 2007).

dndD is predicted to encode a protein of 663 amino acid residues with homology to the ATP-binding cassette (ABC) ATP-binding proteins. An ATP/GTP-binding Walker A motif (35-GLNGCGKT-42) and an ABC transporter family signature

Figure 2. *dnd* genes are responsible for DNA degradation phenomena in wild type *S. lividans* 1326. (A) Five *dnd* genes (*dndA*–*E*) are involved in DNA phosphorothioate modification in *S. lividans*. (B) During conventional electrophoresis, wild-type *S. lividans* 1326 displayed Tris-dependent DNA degradation. The disruption of individual *dnd* genes, including *dndA* (XTG1), *dndC* (XTG3), *dndD* (XTG4) and *dndE* (XTG5), abolished the DNA degradation. In contrast, the degradation phenomenon was enhanced in the *dndB* mutant XTG2. All degradation in these samples could be inhibited by addition of the reducing reagent thiourea.

(556-LSAGERQLLAISLLW-570) were detected in DndD (Connelly et al., 1998; Zhou et al., 2005). DndD also shows extensive homology to Structural Maintenance of Chromosomes (SMC) family proteins associated with ATPase activity (Connelly et al., 1998). SpfD, a homolog of DndD, is essential for Dnd in *P. fluorescens* Pf0-1. SpfD showed a specific ATPase activity of 6.201 ± 0.695 units/mg protein. Thus, this suggests that SpfD/DndD likely provides the energy for stabilizing DNA secondary structures during the modification process by hydrolyzing ATP and acts as an ATP-modulated DNA cross-linker (Zhou et al., 2005; Yao et al., 2009).

As the smallest Dnd protein, DndE consists of only 126 amino acids. DndE has 46% identity to phosphoribosylaminoimidazole carboxylase (NCAIR synthetase) from *Anabaena variabilis* ATCC 29413, which is known to act at a condensing carboxylation step in purine biosynthesis (Nakamura et al., 2002). However, the biochemical function of DndE has not yet been demonstrated.

Western blot with an antibody raised against DndD protein revealed the native expression of DndD in its wild-type/complementation host. Notably, overdosage of DndC or DndD, but not other Dnd proteins in *S. lividans*, was found to be detrimental to cell viability (Zhou et al., 2005; Xu et al., 2009). This suggested that the expression of the *dnd* gene cluster in the native host is under strict control (Zhou et al., 2005; Xu et al., 2009).

THE *dnd* GENE CLUSTER IS WIDESPREAD IN DIVERSE AND DISTANTLY RELATED BACTERIA

An extensive investigation of bacteria genome sequences reveals that *dnd* gene cluster homologues are widespread in diverse and distantly related bacterial species from various geographic niches, including *E. coli*, *Pseudomonas*, *Salmonella*, *Bacillus*, *Oceanobacter*, *Shewanella*, *Hahella*, *Geobacter*, *Candidatus*, *Pelagibacter*, and *Nostoc*, etc (Zhou et al., 2005; He et al., 2007). These bacteria range from GC-rich *Streptomyces* to AT-rich *Pelagibacter Ubique*, from nonpathogenic *Geobacter* to pathogenic *Salmonella*, from soil-dwelling organisms to marine microbes and to even those from the environmental DNA of the Sargasso sea (Zhou et al., 2005; He et al., 2007).

The *dnd* gene cluster from *Salmonella enterica* serovar Cerro 87 was successfully expressed in *E. coli* and conferred modification to the host DNA and subsequent DNA degradation during gel electrophoresis (Wang et al., 2007). Yao et al. located and characterized the *dnd* gene cluster in another gram-negative bacteria *Pseudomonas fluorescens* Pf0-1 (Yao et al., 2009). Interestingly, the Dnd phenomenon was found in nearly 50% of *Mycobacterium abscessus* isolates (Zhang et al., 2004). A survey on 74 actinomycete strains collected from geographically distinct regions identified five strains that possess the Dnd phenomenon (He et al., 2007).

The studies by He et al. show that the *dnd* gene cluster in

Streptomyces lies in a 93-kb genomic island, which can spontaneously excise from the chromosome at low frequency. The further investigation of 11 sequenced bacterial genomes harboring *dnd* gene cluster demonstrated that all of the *dnd* gene clusters lie on mobile elements, 10 of which are located in genomic islands, and one in a large plasmid. This suggests that the *dnd* gene clusters were organized into a functional locus on a mobile element in very ancient times, prior to extensive spread and sequence diversification over the eons (He et al., 2007).

CHEMICAL NATURE OF Dnd MODIFICATION

The precise chemical nature of Dnd modification is essential to fully understand this unusual DNA modification. However, it has been a long-lasting mystery from the beginning (Zhou et al., 1988). The low abundance of the modified species, possible co-elution with normal bases during chromatography, and/or chemical instability have obstructed its elucidation (Boybek et al., 1998; Zhou et al., 2005).

The putative functions of two *dnd* genes (*dndA* and *dndC*: cysteine desulfurase gene and ATP pyrophosphatase gene homologs) associated with sulfur transfer and showed homology to two counterpart enzymes, IscS and ThiI (Mueller et al., 1998; Lauhon and Kambampati, 2000), associated with tRNA sulfur modification. This prompted ^{35}S feeding experiments, which led to the discovery of incorporation of sulfur into DNA from Dnd⁺ strains, including *S. lividans* 66, *S. avermitilis* NRRL8165, and *P. fluorescens* Pf0-1 (Zhou et al., 2005). Unfortunately, the assumption from previous work that the modification might occur on guanine bases (Boybek et al., 1998; Dyson and Evans, 1998) misled the initial stage of the identification of the sulfur modified DNA molecule.

The defined chemical structure of this unusual DNA modification is rather surprising: bacterial *dnd* gene clusters incorporate sulfur into the DNA backbone as a sequence-selective and stereo-specific phosphorothioate modification (Wang et al., 2007). Unlike any other DNA or RNA modification systems, DNA phosphorothioation by the *dnd* gene clusters is the first physiological modification uncovered, which acts on the DNA backbone (Fig. 1) (Wang et al., 2007).

Wang et al. fed *E. coli* B7A (harboring a natural set of *dnd* gene cluster) and DH10B (pJTU1238) (carrying the *dnd* gene cluster from *Salmonella enterica* serovar Cerro 87) with ^{35}S L-cysteine to label the modified DNA molecule. Hydrolyzed and dephosphorylated nucleosides were resolved by reversed phase HPLC followed by scintillation counting to localize ^{35}S -containing fractions. The subsequent mass spectrometric analysis of the ^{35}S containing DNA molecules revealed a characteristic m/z value of 597.1385, proven to be a phosphorothioate diester linkage between dG and dA dinucleotides d(G_{PS}A) in which the nonbridging oxygen is replaced by sulfur (Wang et al., 2007).

Distinct from *E. coli* B7A, *S. lividans* 1326 bears phosphor-

othioate modification between two dGs (d(G_{PS}G)), consistent with the observation of Tris electrophoresis-sensitive cleavage between guanines in GGCC motifs in DNA from *S. lividans* (Fig. 1) (Boybek et al., 1998; Dyson and Evans, 1998; Liang et al., 2007). This demonstrates that DNA phosphorothioate modification acts with sequence selectivity in different species. Moreover, the natural phosphorothioate modification in d(G_{PS}A) and d(G_{PS}G) both occur in *R_P* configuration, which suggests that phosphorothioation of DNA by the *dnd* modification systems is stereo-selective (Wang et al., 2007).

To address the correlation between the Dnd phenomenon and phosphorothioate modification, native (from *E. coli* B7A) and synthetic d(G_{PS}A) were subjected to an *in vitro* Tris-dependent DNA cleavage assay with activated Tris buffer. Notably, degradation of d(G_{PS}A) was observed, generating dG and dA. In contrast, d(G_PA) with unmodified phosphodiester bond remained intact when treated with activated Tris, demonstrating that the phenomenon of DNA degradation during electrophoresis is caused by the site-specific attack on the phosphorothioate modified DNA backbone (Wang et al., 2007).

IMPLICATIONS AND PERSPECTIVES

Great progress has been made since the first observation of the Dnd phenomenon. An unprecedented DNA modification was unveiled: phosphorothioation between two deoxynucleotides within conserved "core" sequence surrounded by necessary flanking sequences. Particularly, Dnd proteins cooperate to sequence-selectively and stereo-specifically replace the oxygen on the DNA backbone with sulfur. Only two enzymes, IscS and Thil, are needed for the biosynthesis of 4-thiouridine (s⁴U) in tRNA (Mueller et al., 2001). IscS converts L-cysteine to L-alanine and sulfane sulfur in the form of a cysteine persulfide. The sulfane sulfur generated by IscS is transferred sequentially to Thil and then to tRNA during the *in vitro* synthesis of s⁴U (Kambampati and Lauhon, 2000). In contrast, DNA phosphorothioation is associated with five proteins and potential DNA secondary structures, suggesting that the biochemical pathway of phosphorothioation is much more complicated than the biosynthesis of s⁴U. Although the biochemical activity of several Dnd proteins has been assayed, additional insights are still needed to elucidate the role of each Dnd protein in the DNA phosphorothioation pathway and the interaction between Dnd proteins and target DNA regions. The *dnd* gene cluster is widespread in diverse distantly related bacteria, however, a complete set of *dnd* homologs has not yet been found in mammals.

Phosphorothioates have been chemically synthesized for decades and incorporated into DNA *in vitro* by enzymatic polymerization using deoxynucleoside α-thiotriphosphates (Brody and Frey, 1981). There are a number of applications of phosphorothioates, including DNA sequencing (Gish and Eckstein, 1988), site-directed mutagenesis (Olsen and Eckstein, 1990), and antisense therapy (Stein, 1996), as

well as potential antiviral activity against human immunodeficiency virus (HIV) via inhibition of de novo viral DNA synthesis and HIV reverse transcriptase activity *in vitro* (Matsukura et al., 1987). The replacement of oxygen by sulfur confers nuclease resistance to phosphorothioated DNA and oligos, providing extensive applications in antisense therapy. Currently, several phosphorothioate oligos have proceeded to phase II/III trials as therapeutic agents against cancer, Hepatitis C, asthma, and Crohn's disease (Kurreck, 2003). Vetravene, a phosphorothioate oligo, was designed for the local treatment of cytomegalovirus retinitis in patients with AIDS and is the first approved antisense drug in the USA (Kurreck, 2003).

Another important question that remains is that of the physiological function of the phosphorothioation system. The phosphorothioate linkage directly introduced by polymerases is in the *R_P* configuration (Eckstein, 1985), which is identical to naturally occurring phosphorothioates in DNA introduced by *dnd* genes. Thus, most of the characteristics of the *in vitro* enzymatically synthesized phosphorothioate bond, including structural and replicative compatibility, are readily applied to the phosphorothioate bonds generated by *dnd* modification system. Phosphorothioate linkages showed enhanced resistance to enzymatic hydrolysis. For instance, the 3'-exonuclease hydrolysis of the Klenow fragment, snake venom phosphodiesterase, and exonuclease III cleave phosphorothioate linkages of the *R_P* configuration 100-fold slower than phosphate linkages (Burgers and Eckstein, 1979; Gupta et al., 1984; Labeit et al., 1987; Verma and Eckstein, 1998). Particularly, the incorporation of phosphorothioates at the sites of cleavage has inhibitory effect on the activity of a number of restriction endonucleases (Olsen et al., 1990; Verma and Eckstein, 1998). In a word, the known resistance of phosphorothioate linkages in nucleic acids to a variety of nuclease activities (Potter et al., 1983; Eckstein, 1986) and the postreplicative (Dyson and Evans, 1998) and site-specific (Zhou et al., 1988; Ray et al., 1992; Wang et al., 2007) nature of the modification suggest that phosphorothioation by the *dnd* modification system might play a role similar to DNA methylation (McClelland et al., 1994) in protecting specific DNA against nucleases (Wang et al., 2007).

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