Novel post-replicative DNA modification in *Streptomyces*: analysis of the preferred modification site of plasmid pIJ101

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ABSTRACT

Both *Streptomyces lividans* and *Streptomyces avermitilis* have the ability to site specifically modify their DNA, rendering it susceptible to *in vitro* Tris-dependent double-strand cleavage. We have cloned a 160 bp fragment containing the preferred modification site of plasmid pIJ101 and, employing an *in vitro* primer extension assay, determined that the modifications occur at guanine residues on either strand separated by 3 bp. These guanines are located within a 6 bp palindromic ‘core’ sequence. A cloned copy of a 35 bp region of the plasmid containing this core sequence was not recognized by the modifying activity *in vivo*. To further investigate the nature of the site specifically a set of deletion mutants of the 160 bp sequence were analysed. This revealed that a substantial portion of this sequence is essential for authentic modification. The essential region contains three 13 bp direct repeats, the central one containing the core sequence, while the left-hand and right-hand copies overlap two potential stem–loop structures. Deletion of either left- or right-hand repeat structures abolishes modification within the core sequence, although the left-hand deletion resulted in modification at a secondary site within the right-hand direct repeat. These data support a post-replicative mechanism of modification, underlined by the observation that the modifications are not detected in single-stranded plasmid replication intermediates.

INTRODUCTION

DNA modification serves to increase the number of different bases in the double helix and hence can have profound effects on many aspects of DNA metabolism. A modified base can be synthesized at the mononucleotide level and incorporated during replication by DNA polymerase, as is often the case for many modifications of diverse structure found in bacteriophage genomes (1). Alternatively, specific proteins can carry out enzymatic modification of a normal base at the polynucleotide level. Classical DNA modification in bacteria is of this type, involving methylation of either cytosine or adenine residues, and, due to a requirement for specific recognition of the substrate DNA sequence by the modification enzyme, there is inherently more site specificity in the positioning of modifications introduced by this process (2).

We have been investigating a non-classical DNA modification found in the DNA of two Gram-positive *Streptomyces* species. The modification is found in *Streptomyces lividans* (3,4), the member of the genus most widely used for genetic manipulation, and *Streptomyces avermitilis* (5), a producer of commercially important antihelmintic secondary metabolites called avermectins. In both species the modified base is estimated to constitute 0.1% of the total base composition. To investigate its biological significance an *S.lividans* mutant, ZX1, lacking the modification has been isolated (4) and the corresponding gene, dndA, isolated (X.Zhou and T.Kieser, personal communication). Although the chromosome of ZX1 contains a large deletion (6), a precise *dndA* mutant was constructed by gene replacement in the wild-type. Preliminary analysis of this mutant suggests that it is affected in the level of expression of certain cloned genes (X.Zhou and T.Kieser, personal communication).

To date it has not proved possible to purify the base to determine its structure (4; P.Dyson, unpublished results). This may in part be due to its low abundance, but could also be a consequence of co-elution during chromatography with one of the standard bases or as a result of its susceptibility to chemical modification and destruction during isolation. The known *in vitro* reactivity of the modification does imply that it is probably unlike any other which has been previously characterized. The modifications react with a peracid derivative of Tris which is formed at the anode during conventional gel electrophoresis (7,8). Concerted peracid-mediated oxidative and amine-catalysed reactions result in strand cleavage at the site of modification. The reaction products as observed on gels are discrete DNA fragments, resulting from Tris-dependent cleavage at closely opposed site-specific modifications. Indeed, evidence from running electrophoretically cleaved DNA on alkaline gels, which would reveal single-stranded (ss) nicks, suggests either that single strand-specific modification is uncommon or that modifications on one strand alone are not susceptible to the cleavage reaction (4).

There is variance in the frequency of Tris-dependent double-stranded (ds) cleavage at different sites, which probably reflects differences in modification frequency at these locations; it is estimated that only up to 50% of high frequency sites on multi-copy genomic sequences are modified. The location of these sites can be determined by restriction mapping in conjunction with Tris-dependent cleavage and a map of 14 high frequency modification...
sites in plasmid pIJ303 has been constructed (5). The position of the preferred modification site in the related plasmid pJJ01 was also determined by end-labelling at the site of cleavage with polynucleotide kinase and performing Maxam–Gilbert sequencing reactions on the product (4). Although the precise site of modification and cleavage could not be determined in this way, as the smallest labelled fragments tend to be lost prior to gel loading, it was estimated to be ~20 bp distant from a Smal site (position 8461 in the published sequence of pJJ01; 9). The readable sequence ladder obtained implied that a unique end was created by the cleavage reaction.

We have now examined this preferred modification site in more detail to precisely determine which bases are modified. By deletion analysis we have also demonstrated that authentic modification requires a considerable extent of flanking sequence, including sequences with potential for secondary structure formation. Both the site specificity and the important role of distal flanking sequences in determining modification strongly suggest a post-replicative mechanism and this is supported by the finding that the modifications are under-represented in ss plasmid replication intermediates.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

*Escherichia coli* strain F− Z− AM15 (10) was routinely employed as a host for plasmids used and constructed in this study. DNA modification was assayed after transformation and re-isolation of plasmid DNA from *S. lividans* 66 (provided by D.A. Hopwood, John Innes centre, Norwich, UK), *S. lividans* ZX1 (4) and *S. avermitilis* 12804 (National Collections of Industrial and Marine Bacteria, Aberdeen, UK). The plasmids employed in the study were pUC18 (11), pBGS19 (12), pJD19 and pUCS75 (13), pHe99 (14) and pJJ02 (15).

**Media, transformation and growth conditions**

*Escherichia coli* strains bearing recombinant plasmids were cultured on L broth or plated on L agar (16) supplemented with appropriate antibiotics (25 µg/ml kanamycin, 50 µg/ml ampicillin, 10 µg/ml gentamicin). Recombinant pUC18, pBGS19, pJD19 and pUCS75 derived plasmids were screened on media containing 20 µg/ml Xgal. Plasmids were introduced by transformation using a standard calcium chloride-mediated procedure (16). For plasmid DNA isolated from *Streptomyces*, however, a HEPES-based buffer (7) was employed to avoid Tris-dependent DNA cleavage. For Southern hybridization DNA was transferred to Hybond N membrane (Amersham) using a vacuum blotting system (Stratagene). A pJJ02 probe was prepared by labelling with digoxigenin-11-dUTP by random priming using a kit from Boehringer Mannheim. Hybridization conditions and subsequent detection by colour reaction with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were according to the manufacturer’s instructions (Boehringer Mannheim).

**Plasmid constructs**

The 160 bp fragment carrying the preferred modification site of pJJ01 was originally cloned as a *KpnI–ApaI* fragment into *KpnI* and *HincII*-cleaved pUC18. To do this pH699 DNA was digested with *ApaI*, treated with SI nuclease to remove the overhanging 5′-tails and then cut with *KpnI*. One of the recombinant plasmids recovered, pUCS49, was created by fusion of the blunt-ended *KpnI–ApaI* 160 bp fragment with a 21 bp *HincII–KpnI* linker, derived from the pUC18 polylinker, together cloned into the *KpnI* site of pUC18. This plasmid was used as the source to subclone a 232 bp *EcoRI–HindIII* fragment containing the modification region into the polylinker of the *Streptomyces–E.coli* shuttle vector pUCS75, to create pUCS82. The orientation of this region was subsequently flipped by subcloning a 166 bp *KpnI–XbaI* fragment from pUCS82 into *KpnI* and XbaI-digested pUC18, to create pUCS94. A 202 bp *EcoRI–HindIII* fragment was subcloned from this plasmid into pUCS75 to generate pUCS101. To remove 32 bp of the left-hand (LH) flanking sequence a 128 bp *XbaI–Smal* fragment was subcloned from pUCS94 into *XbaI* and *Smal*-cleaved pBGS19, to generate pUCS100. An 168 bp *EcoRI–HindIII* fragment containing the 128 bp truncated modification region was then subcloned from pUCS100 into pUCS75 to create pUCS102. To remove a further 27 bp of LH flanking sequence pUCS100 was first cleaved with *SacII*, treated with SI nuclease to remove the 3′-overhang and then cut with *XbaI*. The modification region contained within a 101 bp fragment was then cloned into pJD18 cut with *XbaI* and *Smal*, to create pUCS114, and from there as a 141 bp *EcoRI–HindIII* fragment into pUCS75, to generate pUCS118. To delete 54 bp of right-hand (RH) flanking sequence pUCS100 was digested first with *BstWI*, then digested with SI nuclease to remove total genomic DNA was isolated from *S. lividans* by neutral lysis, as previously described (21). Biomagnetic separation was used to further purify pJJ02 ssDNA. An aliquot of 250 pmol 39mer 5′-biotinylated oligonucleotide, complementary to the (+) strand of pJJ02 in the region 76–115 bp upstream of the preferred modification site and immediately adjacent to the *KpnI* site, of sequence 5′-bio-ACGGC GCACGTCTGCTGAGGCGGAGTG- CGCGCGGCGAG was immobilized to 1 mg Dynabeads M-280 Streptavidin according to the manufacturer’s instructions (Dynal). A 700 µl aliquot of isolated DNA, containing an estimated 10 µg pJJ02 ssDNA, was combined with 1 mg Dynabeads with the immobilized oligonucleotide. This and subsequent steps to purify the ssDNA were performed according to the manufacturer’s instructions, to yield ~5 µg ssDNA. To obtain the (+) strand from pJJ02 dsDNA the same purification procedure was used after denaturing 10 µg Wizard column-purified plasmid at 95°C for 10 min.

DNA was routinely visualized after electrophoresis in 0.8–1.2% agarose gels prepared in TBE buffer (16). For plasmid DNA isolated from *Streptomyces*, however, a HEPES-based buffer (7) was employed to avoid Tris-dependent DNA cleavage. For Southern hybridization DNA was transferred to Hybond N membrane (Amersham) using a vacuum blotting system (Stratagene). A pJJ02 probe was prepared by labelling with digoxigenin-11-dUTP by random priming using a kit from Boehringer Mannheim. Hybridization conditions and subsequent detection by colour reaction with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were according to the manufacturer’s instructions (Boehringer Mannheim).

**DNA isolation and manipulation**

Plasmid DNA was isolated from *E.coli* by the alkaline lysis technique (20) and typically from *Streptomyces* by a modified version of this technique (17). Prior to sequencing and primer extension assays the DNA was further purified by precipitation. The DNA was then resuspended and further processed using Wizard plasmid purification columns according to the manufacturer’s instructions (Promega). In order to isolate native pJIJ02 ssDNA
the 5′-overhang, then cut with Hincll. The plasmid was then re-ligated to create pUCS115, from which a 108 bp EcoRI–HindIII fragment containing the truncated modification region was subcloned into pUCS75 to generate pUCS119. The smallest 35 bp modification region which was analysed comprised a linker prepared by annealing two complementary oligonucleotides of sequence 5′-CGCGGTTCGACCCGCCGCCGGTGCGC- GTACG and 5′-GATCCGTACGGGGCAGCGCGGCGCG- GTCGAAGCGCGAGCT. The linker was inserted between the SacI and BamHI sites in the polylinker of pUC18, to create pUCS113, before subcloning it as a 71 bp EcoRI–HindIII fragment into pUCS75, to generate pUCS120. To clone the same region in the opposite orientation the oligonucleotides 5′-GATCCG- CGCTTCGACCCGCCGCCGGTGCGC- GTACG and 5′-CGTACGGCGAAGCGCGGAGGTCGAAAGCG- GCC were annealed and cloned between the SacI and BamHI sites of pUC18, to make pUCS112, before subcloning the 71 bp EcoRI–HindIII fragment into pUCS75, to create pUCS117.

Tris-dependent DNA cleavage and primer extension assays

The nucleolytic activity was generated in standard Tris–acetate–EDTA (TAE) buffer containing 40 mM Tris, 20 mM sodium acetate, 0.8 mM EDTA, pH 7.5, adjusted with acetic acid. Conditions were employed which have been previously demonstrated to cause maximal DNA cleavage in the presence of saturating amounts of the nucleolytic activity (8). A 250 µl sample of buffer, maintained at 37 °C, was electrophoretically activated by placing it in a horizontal submarine gel chamber (BioRad) with platinum electrodes and applying a constant voltage of 80 V. After 10 min activation 500 µl of the buffer was sampled from –5 mm adjacent to the anode and added to 0.5 µg DNA in 5 µl H2O in a microfuge tube. The cleavage reaction was then allowed to proceed for 2 h at 37 °C. The reaction was terminated by addition of 50 µl 3 M sodium acetate, pH 5, and 550 µl isopropanol. The DNA was precipitated at –20 °C prior to centrifugation, drying and resuspension of the pellet in 10 µl H2O.

Primer extension and DNA sequencing reactions were performed with a Circum Vent Thermal Cycle Dideoxy DNA Sequencing Kit largely according to the manufacturer’s instructions (New England Biolabs). For reactions on templates cloned in pUCS75 standard 24mer forward and reverse sequencing primers were used. For pIJ702 templates the primer was a non-biotinylated version of the same 39mer as used for purification of ssDNA by biomagnetic separation. Primers were 5′-end-labelled with [γ-32P]ATP and T4 polynucleotide kinase. In primer extension reactions a deoxynucleotide mix consisting of 30 µM dATP, 100 µM dCTP, 100 µM dGTP and 100 µM dTTP substituted for the deoxy/dideoxy sequencing mixtures provided in the kit. For thermal cycling a ‘hot-top’ Crocodile III thermal cycler (Appligene) was employed, using 20 cycles of 30 s denaturing at 95 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C. Extension and sequencing products were separated using 8% polyacrylamide denaturing sequencing gels prepared by appropriate concentration of Tris and Tricine. Autoradiographs were made by exposing X-ray film (Hyperfilm βMax; Amersham) against the gel at room temperature. They were then scanned with an HP ScanJet IICX and the signal intensity of specific primer extension bands quantified using Phoretix Life Software (Phoretix Ltd, Newcastle, UK). Loading variations between lanes were corrected according to the signal intensities of reference bands not arising due to Tris-mediated cleavage of the template. As an over-saturated exposure of the film could be formed due to termination of primer extension opposite positions of high frequency Tris-mediated cleavage, a series of shorter exposures was made to obtain a reliable estimate of the amounts of these products. The results presented represent mean values obtained from three independent DNA cleavage reactions on modified DNA (standard deviations were ≤14% of these values).

RESULTS

Sequence determination of the preferred modification site of pIJ101

To determine the precise location of the preferred site of modification in pIJ101 we subcloned a 160 bp KpnI (position 8429)–Apal (position 8591) fragment into the shuttle vector pUCS75 (13). The resulting recombinant plasmid pUCS101 was passaged through S.lividans. Plasmid DNA was subsequently purified and an aliquot treated with activated Tris. Both Tris-treated and untreated DNA were employed in primer extension reactions using a pUC/M13 forward primer. For untreated template with normal dNTPs in the reaction some termination of primer extension was observed in the vicinity of where modification was expected (Fig. 1). However, similar polymerase stalls were observed if the template was purified from E.coli, suggesting that it is unrelated to DNA modification and probably attributable to DNA secondary structure. The DNA reacted with Tris exhibited a very strong specific chain termination signal opposite a guanine on the template strand (located at position 8506 in pIJ101). Likewise, reverse primer extension with the Tris-reacted and modified DNA gave a specific product opposite a guanine at position 8505 (results not shown). These guanine residues are located within a 6 bp palindromic sequence (Fig. 2). The amounts of the specific

Figure 1. Determination of the position of modification by forward primer extension using pUCS101 as template. pUCS101 DNA from S.lividans was employed as template for dideoxy sequencing reactions (lanes T, G, C and A) and for primer extension with all four standard deoxynucleotides (lane –). An aliquot of the same DNA was incubated with activated Tris prior to primer extension with deoxynucleotides (lane +) and a C-specific dideoxynucleotide reaction (lane +/C). The arrow indicates the position of the specific primer extension product due to Tris-mediated cleavage of the template at the position of base modification.

flanking sequences present in the 160 bp modification region. For investigating this we constructed deletions of both LH and RH region surrounding the central 6 bp palindrome as substrate. To sequence, the modification system required a more extensive shown). This suggested that, irrespective of the orientation of this mutant ZX1 (4) there was no evidence for specific termination of pUCS101 DNA passaged through the cloned in pUCS117, was also not modified with Tris-reacted templates (Fig. 3). A second synthetic sequence, extension assays we could detect no specific termination products template and employing both forward and reverse primer. However, using the resultant plasmid pUCS120 as template after in vitro Tris-mediated strand scission similar amounts of extension products were observed, terminating at the same positions as for the S.lividans DNA (results not shown). For pUCS101 DNA passaged through the S.lividans DNA modification mutant ZX1 (4) there was no evidence for specific termination of primer extension due to Tris-mediated DNA cleavage (results not shown).

Defining a minimal modification site

The site specificity of modification is consistent with a post-replicative mechanism. By analogy with classical modification systems, we expected that the modifying activity would recognize a specific target sequence and modify bases within this sequence. To address this we proceeded to clone a 35 bp synthetic sequence, which included the central 6 bp palindrome together with a short amount of sequence to either side, to test if it would be modified in vivo. However, using the resultant plasmid pUCS120 as template and employing both forward and reverse primer extension assays we could detect no specific termination products with Tris-reacted templates (Fig. 3). A second synthetic sequence, with the 35 bp modification site in the opposite orientation and cloned in pUCS117, was also not modified in vivo (results not shown). This suggested that, irrespective of the orientation of this sequence, the modification system required a more extensive region surrounding the central 6 bp palindrome as substrate. To investigate this we constructed deletions of both LH and RH flanking sequences present in the 160 bp modification region. For each deletion mutant constructed, modification was assayed using the primer extension assay after purification of plasmid DNA passaged through S.lividans. The results are summarized in Figure 3 and revealed that in vivo modification requires a LH flanking sequence >13 bp and flanking sequence >14 bp and ≤83 bp. Interestingly, for plasmid pUCS118, which contains a complete RH flanking sequence but only 13 bp of proximal LH sequence, a forward primer extension product was observed resulting from termination opposite a Tris-reacted cleavage site at position +31 with respect to the pUCS101 product (equivalent to position 8537 in pIJ101). Using the reverse primer a specific termination product was found opposite position –33, again with respect to the pUCS101 product (position 8538 in pIJ101). Moreover, the amount of termination detected opposite guanines within the 6 bp palindrome was not significantly greater than for the control lane, indicating little or no Tris cleavage at these positions in this deletion mutant. The amount of each of the termination products at the displaced location in this mutant were quantified to indirectly assess the degree of modification. This indicated that modification was 10-fold less efficient at this secondary site compared with authentic modification within the 6 bp palindrome of the full-length clone. Examination of this secondary sequence showed that it shared homology with the sequence encompassing the authentic site (Fig. 2). Further scrutiny of the sequences revealed that the complete 160 bp fragment in fact contains three 13 bp imperfect direct repeats of this sequence, as well as two long inverted repeats of different sequence in each flanking sequence. The direct repeats overlap the arms of the inverted repeats and complete deletion of either inverted repeat together with the overlapping direct repeat resulted in no modification within the 6 bp palindrome in vivo (Fig. 3). A partial deletion of the LH direct and inverted repeats, as represented by plasmid pUCS102, did not affect positioning of the ds modifications, but caused a reproducible reduction in their frequency compared with the full-length clone.
Modification is not detected in single-stranded plasmid replication intermediates

The unusual extent of the minimal modification site suggested that these repeat structures could either be recognized by the modifying activity or involved in the region adopting a particular secondary structure which could be a substrate for modification. Formation of such structures could be facilitated in unpaired ss plasmid replication intermediates. By examining if these ss molecules are modified we could establish if the modification system operates (i) either at the mononucleotide level or on ss replication intermediates or (ii) following replication on a ds substrate. To perform this analysis we employed a pIJ101 derivative, pIJ702, which lacks the primary site for second strand synthesis. As a consequence, replication results in accumulation of the (+) strand (the displaced strand) in the cell (22–24). These ss molecules are especially abundant in young, rapidly dividing cultures, indicating a high turnover of plasmid replication. The profile of plasmid DNA purified by neutral lysis from these cultures showed a large amount of ssDNA together with a range of different negatively supercoiled topoisomers of the ds plasmid (Fig. 4A). We selectively purified the ssDNA using biomagnetic separation. To test if modified pIJ702 ssDNA is susceptible to Tris-mediated strand scission the (+) strand was also obtained in a similar manner from heat-denatured pIJ702 dsDNA purified by alkaline lysis (in contrast to preparations obtained by neutral lysis, the majority of this DNA was fully supercoiled together with some nicked, open circular DNA). The yield and quality of these ssDNA preparations were monitored by Southern hybridization, comparing blots obtained with and without alkali denaturation of gels, by which ssDNA can be readily discriminated. DNA samples were then reacted with activated Tris and primer extension reactions were subsequently performed with a primer complementary to the (+) strand, which binds 5′ of the preferred modification site. Analysis of the primer extension products revealed no evidence for modification in the ss replication intermediate, whereas both pIJ702 dsDNA and the heat-denatured (+) strand derived from this gave strong termination signals at the expected positions (Fig. 4). The latter result indicated that modified ssDNA is susceptible to Tris-mediated cleavage.

DISCUSSION

The preferred modification site of pIJ101 is located within an intergenic region between the rep gene and two divergent ORFs (85 and 79) of unknown function. Tris-mediated cleavage is believed to involve β-elimination of both phosphates from the sugar of the modified base, releasing the template fragment with a 5′-phosphate group (8). As extension from either forward or reverse primers terminates opposite the second guanine in a GpG sequence, the inference is that it is the first guanine in the dinucleotide on either strand which is modified in vivo and which is susceptible to in vitro strand scission. These guanines are located 3 bp apart within a 6 bp palindrome. Our preliminary evidence from sequencing other modification sites also indicates that closely opposed guanine residues are modified. We are aware of only one other reported naturally occurring modification of guanine: 0.3% of guanines in the Shigella dysenteriae phage DDV1 are believed to be methylated by a phage-specific methylase to give 7-methylguanine (25). In E.coli this methylated purine is also a major product of exposure to alkylating agents, although it persists for a long time, being inefficiently removed by DNA glycosylase activity (26). Methylation of the N7 position of guanine is also the first step in G-specific chemical sequencing reactions, subsequent strand scission being mediated by piperidine (27). However, as the modifications in S.lividans DNA are not susceptible to piperidine cleavage (4), this indicates that they represent an entirely novel type of natural DNA modification of guanine. Primer extension on intact modified templates is not impeded by the modified base, with incorporation of a complementary cytosine, indicating that normal base pairing occurs, as would be expected for a natural DNA modification as opposed to a lesion.

We could not detect modification of ss plasmid replication intermediates, even though this DNA represents the displaced strand of ds molecules. This clearly indicates that the modifying activity acts post-replicatively on unmodified dsDNA substrates. Moreover, it implies that when there is a high turnover of plasmid replication in young cultures the modifying activity is inefficient at modifying transient ds molecules. It has been previously noted that during lytic growth of actinophage ΦC31 in S.lividans the phage DNA is not modified (4). Classical post-replicative modification can normally keep pace with rapid phage DNA synthesis, resulting in methylation of phage DNA. The Streptomyces modification system would appear inefficient in comparison, underlined by the observation that modification of genomic DNA, even from stationary phase cultures, is only partial. The absence of modification in phage and rapidly replicating plasmid dsDNA may reflect a requirement for the system to operate on a particular DNA topology.

Perhaps the most surprising observation concerns the extent of the modification site. By analogy with classical methylation we were expecting site specificity to be conferred by the 6 bp palindrom and, if not, by very short additional information to
either side. In contrast, our findings indicate a requirement for a far greater extent of sequence information for modification to occur. Deletion mutants in which sequences are removed distal to the core sequence and which, as a result, no longer undergo modification provide further evidence for a post-replicative modification process. Authentic modification requires extensive sequences to either side of the palindromic ‘core’ and this argues against a simple model involving binding of the modifying activity to a distal recognition sequence prior to modification within the core, by analogy with Type I restriction enzyme activity. Each of the flanking sequences in the full-length clone include inverted repeats with potential for stem–loop formation.

Activity. The emerging picture, then, is that determination of site specificity is considerably more complex than for classical post-replicative modification. It may involve interactions between more than one protein and, as well as being sequence specific, could require adoption of DNA secondary structures which would be dependent on DNA topology. To investigate these possibilities we are currently constructing further mutations in this region and also comparing this modification sequence with preferred modification sites cloned from other molecules.

REFERENCES