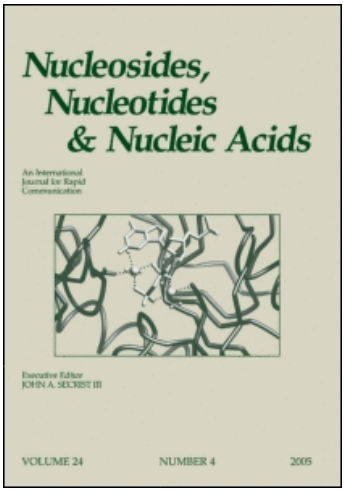


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Activated DNA- And RNA-Duplexes Covalently Cross-Linked to Regulatory Proteins

Zoe A. Shabarova[a]
[a] Chemistry Department, A.N. Belozersky Institute of Physicochemical Biology Moscow State University, Moscow, Russia

To cite this Article Shabarova, Zoe A.(1998) 'Activated DNA- And RNA-Duplexes Covalently Cross-Linked to Regulatory Proteins', Nucleosides, Nucleotides and Nucleic Acids, 17: 9, 2063 — 2072
To link to this Article: DOI: 10.1080/07328319808004747
URL: http://dx.doi.org/10.1080/07328319808004747

NUCLEOSIDES & NUCLEOTIDES, 17(9-11), 2063-2072 (1998)

ACTIVATED DNA- AND RNA-DUPLEXES COVALENTLY CROSS-LINKED TO REGULATORY PROTEINS

Zoe A. Shabarova *

Chemistry Department, A.N. Belozersky Institute of Physicochemical Biology, Moscow State University, 119899 Moscow, Russia

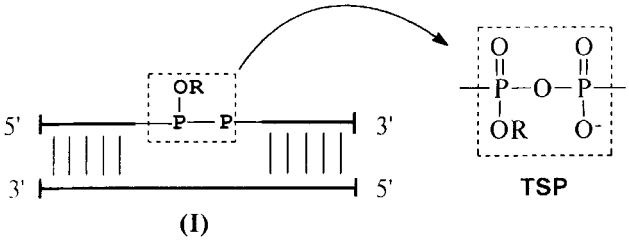
ABSTRACT. A novel class of DNA- and RNA duplexes including trisubstituted pyrophosphate internucleotide groups (TSP) in sugar-phosphate backbone was employed in cross-linking to proteins. Substrates including TSP effectively bound covalently to nucleofilic aminoacids (Lys, His) of transcription factors HNF1 and NF-kB and to HIV-1 Tat protein without additional activation at physiological conditions.

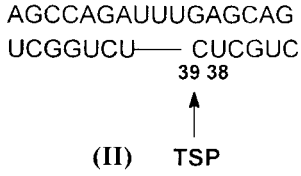
INTRODUCTION

Antisense and antigene strategies provide a useful tool for regulation of gene expression at the transcription level. The decoy or sense approach represents an alternative use of synthetic oligonucleotides for the regulation of gene transcription. In this strategy, oligonucleotde duplexes with recognition sites for the specific DNA- or RNA-binding transcription factors are used as competitive or better incompetitive inhibitors to bind to target proteins and thus to modulate gene transcription controlled by these factors.

In the present communication we describe the synthesis by chemical ligation procedure [1-3] and the use of a new class (I) of chemically active dsDNA derivatives for cross-linking to proteins. A particular property of all these reagents is a spotted distribution of chemically active TSP groups which are able to interact with nucleophilic groups of protein amino acids (Lys, His).

*FAX: (095) 939-3181; E-mail: zoeshab@nuclacid.genebee.msu.su



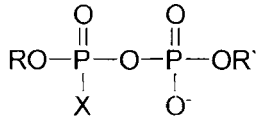


These sequences might be recognised by the protein active sites responsible for protein-nucleic acid interactions, and cross-links emerge directly at these active sites, in zero distance. Region-specific chemical cross-linking of compounds (I) to transcription factors HNF-1 [4] and NF-kB p50 subunit [5] has been successfully performed.

A model HIV-1 TAR RNA duplex containing active groups was synthesised [6]. TSP group incorporated at positions 38-39 in (II) reacted specifically with Lys51 in the basic region of Tat peptide (37-72) [6].

RESULTS AND DISCUSSION

A new class of oligonucleotides with active groups in sugar-phosphate backbone was constructed in MSU in the end of 80-ies [7, 8]. These oligonucleotides may be used in reactions with proteins. Substances comprising this class contain substituted pyrophosphate (TSP) internucleotide groups:

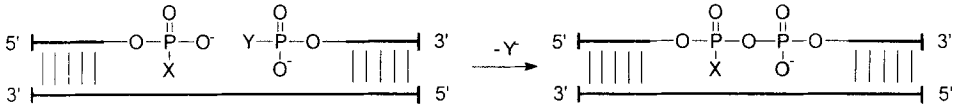


where R, R' are oligonucleotide residues and X- aliphatic alcohol.

The characteristic properties of these substances are: 1) Reactive groups may be introduced in any predetermined position of one of the ds-polynucleotide strands; 2) Modified duplexes are stable in water solutions (pH=6-8) for a long period of time without any substantial loss of reaction activity; 3) Interaction with Lys and His nucleophilic groups of proteins located in nucleic acid-protein complex at a zero distance from a reactive group occurs; 4) Formation of covalently bound complexes with proteins occurs without additional activation at physiological conditions, i.e., at pH 6.0-8.0. Hence, such substances may find broad application in the studies of structure and functions of nucleic acid-recognising proteins and in their affinity modification. Moreover, they may be employed in sense biotechnology and medicine.

TSP may be introduced in a single, definite position in DNA or RNA duplexes

Synthesis of dsDNA fragments containing reactive TSP groups in the definite position of the sugar-phosphate backbone is based on chemical ligation method [1-3] by which two terminal sequences of oligonucleotides are brought together at complementary template and then condensed. In this reaction one of the oligonucleotides carries an aliphatic alcohol or amine residue at its 3'(5') terminal phosphate group while the other one's 5'(3') terminus is phosphorylated [7, 8]. The reaction proceeds according to the following scheme:



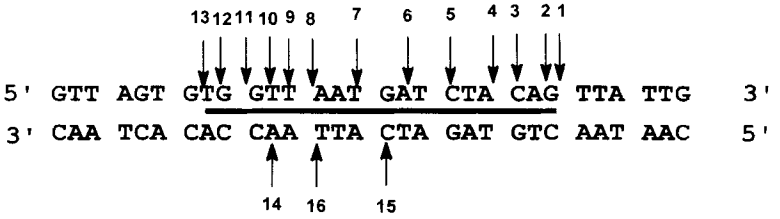
where X is an aliphatic alcohol and Y- a condensation agent residue.

Alkyl esters of oligodeoxynucleotides employed in the reaction are obtained either by postsynthetic modification of 3'-phosphorylated oligonucleotides by alcohols under the action of condensing agents [9] or by automatic oligonucleotide synthesis [10].

The method of chemical ligation was employed to obtain oligodeoxynucleotides with O-alkyl-substituted pyrophosphate residues [7]. A model system with the recognition sites of *Eco*RII, *Sso*II, and *Mva*I restriction endonucleases was analysed. It was demonstrated that the reaction proceeds most effectively when water-soluble carbodiimide (EDC) was used as condensation agent. The yields of chemical ligation process comprised 65-80%, depending on the nature of alkyl residue in TSP.

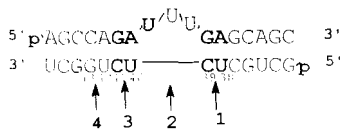
The same approach was used to obtain reactive DNA duplexes containing O-methyl-substituted pyrophosphate group in the *Eco*RII and *Rsr*I recognition sites [11]. In this case, methanol residue was used as non-nucleotide substitute in TSP because it induces small if no changes in the topology of double helix which is essential for future studies of interaction of modified DNA duplexes with proteins.

Proceeding with the experiments, we synthesised a set of DNA duplexes containing O-methyl-substituted pyrophosphate group in various positions of HNF-1 recognition site to study DNA contacts with HNF-1.



(III)

The position occupied by TSP in each of the type III duplexes is indicated by arrows. Active group was introduced by chemical ligation method both in the upper and in the lower strands of the duplexes. Noteworthy, each modified duplex contained only one TSP group in a definite position. DNA duplexes containing TSP internucleotide group in various positions of NF-kB recognition site were obtained by the same method [5]. Introduction of TSP group into the RNA molecules was described in [6, 10]. To study interaction of TAR RNA with Tat protein of HIV-1 virus a set of RNA duplexes containing TSP group in various Tat protein-recognising regions (indicated by arrows) were synthesised.



5' GCUGC(dU)pOEt p*CUCUGGCU position 1
5' GCUGCU(dC)pOEt p*UCUGGCU -"- 2
5' GCUGCUC(dU)pOEt p*CUGGCU -"- 3
5' GCUGCUCUC(dU)pOEt p*GGCUAC -"- 4
3' d(CACGACGAGAG--A----------CCGA[TGA])

It was found that chemical ligation of oligoribonucleotides gave very low yields (1-2%) and thus oligoribonucleotides with 3'-O-alkylphosphate of 2'-deoxynucleozide at 3'-terminus were used to introduce TSP group into RNA duplexes. Moreover, to obtain RNA duplexes ligation was carried out at oligodeoxyribolucleotide template, then the modified strand was isolated and equimolar quantity of complementary oligoribonucleotide was added.

Chemical cross-linking of DNA and RNA duplexes containing active substituted pyrophosphate groups with proteins

Duplexes with trisubstituted pyrophosphate bond possess a unique property: They react selectively with nucleophiles, such as primary and secondary amines, at the twosubstituted phosphate atom leaving the neighbouring one free in the form of an alkylnucleoside phosphate-anion of strong acid.

We suggested to employ this reaction of modified dsDNA substrates for cross-linking with proteins that recognise particular DNA sites.

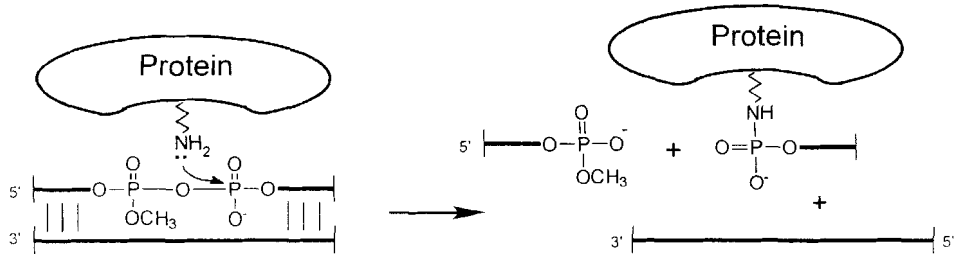


TABLE. Efficiency of cross-linking of some enzymes with oligonucleotides carrying trisubstituted pyrophosphate internucleotide bond

##	Enzyme	Yield of cross-linking product, %	Reference
1	*Eco*RI, restriction endonuclease	1	11
2	*Eco*RI, methylase	35	11
3	*Rsr*I, restriction endonuclease	1	11
4	*Rsr*I, methylase	30	11
5	*Eco*RII, restriction endonuclease	15	12, 13
6	SsoII, restriction endonuclease	20	14

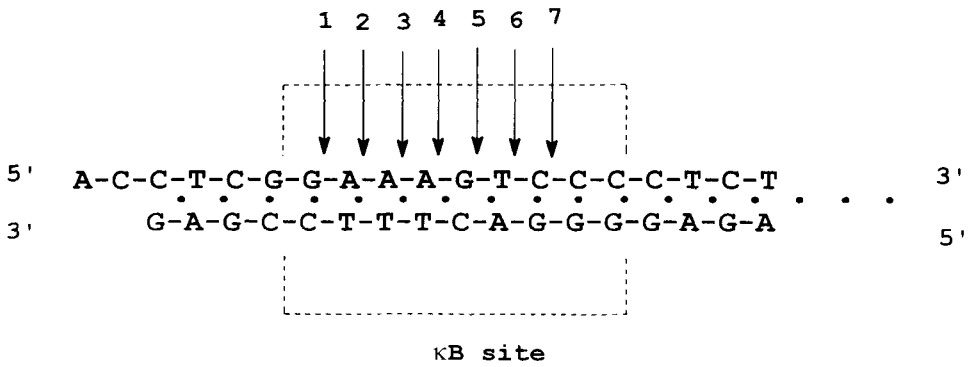
The first results were obtained using restriction-modification endonucleases [11-14].

As shown in the Table, in some cases the efficacy of cross-linking was very high.

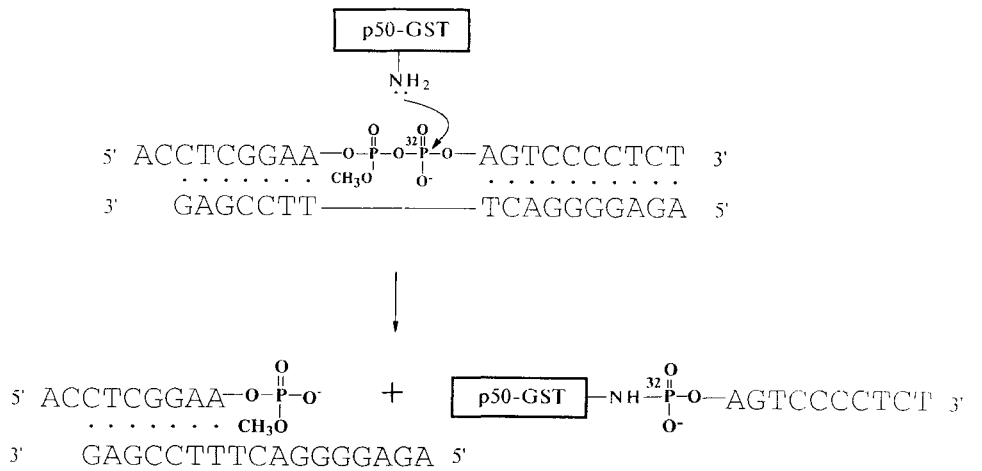
Transcription factors occupy a prominent place in the studies of gene expression. Transcription is inhibited by short, double-stranded oligonucleotides containing HNF-1 recognition sites [15]. To enhance this effect we suggested to use type I DNA duplexes containing active TSP groups. Probing of the active centre of HNF-1 by using a number of DNA duplexes (III) containing O-methyl-substituted pyrophosphate group in various positions (1-13) of HNF-1 recognition site is described in [4]. These experiments were performed either with gene-engineered protein, i.e., with DNA-binding domain of HNF-1, or with nuclear proteins of rat liver. Modified substrates were incubated with the protein or with the nuclear extract at 4 C or at 20° C for 10° min or 12 h. A covalently bound complex was obtained in both options of the reaction, but it was formed only if DNA duplex containing TSP group in position 10 was taken. The yield of covalent binding reaction was up to 40% of the efficacy of non-covalent binding. Covalent binding of modified substrate to HNF-1 of nuclear extract was shown to be specific and other components of nuclear extract were not modified. Hence, oligonucleotides with TSP groups may be successfully used in *in vivo* systems, and, basing on such substances, drugs of a new generation may be elaborated.

To select the optimal structure of inhibitors, another transcription factor, NF-kB, which participates in the expression of the genes of the light chains of immunoglobulins was studied. Many genes possess NF-kB binding sites, and the factor itself is present in all the mammalian cells. Many viruses, including HIV, employ NF-kB to activate their genomes.

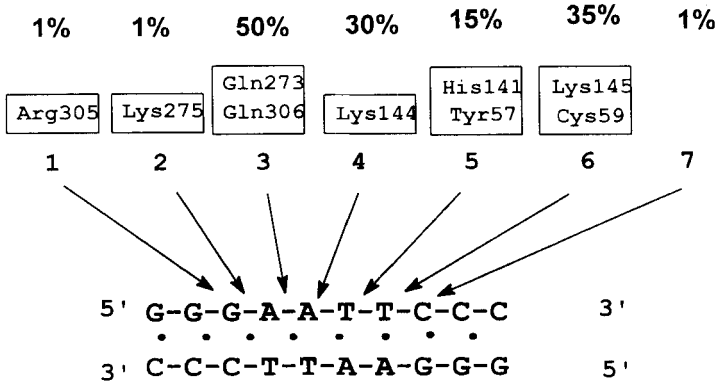
In the next figure the structure of dsDNA with kB-site is given. Arrows indicate the positions in the sugar-phosphate backbone in which the active TSP groups were introduced.



Further, we studied interaction of each of the DNA duplexes with one and the same primary structure, but differing in the position of TSP in the kB region, with p50 subunit of NF-kB [5]. It follows from the X-ray analysis [16-18] of p50 dimer-DNA complex with idealised kB region that three lysine residues, Lys 275, Lys 144, and Lys145, and one histidine residue, His 141, participate in nucleic acid-protein contacts. The next scheme illustrates the reaction between ε-amino group of lysine and one of the modified DNA duplexes:



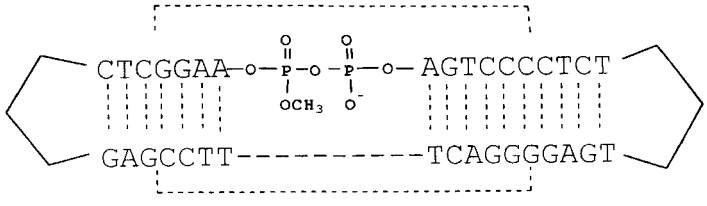
Data on the efficacy of cross-linking (in %%) of duplexes containing TSP groups in positions 1-7 with p50 subunit of NF-kB are presented at next scheme. Square frames denote amino acid contacts revealed by X-ray analysis.



κB site

It follows from the scheme that a relatively high efficacy (30-35%) of covalent binding of duplexes with TSP group in positions 4 and 6 is derived by contacts with Lys144 and Lys 145. Cross-linking with His141 occurs less effectively with TSP in position 5. For a duplex with TSP in position 3, data on contacts obtained by X-ray analysis and on efficacy of cross-linking (50%) are not in accord. One may suppose that there exists no contact of Lys 275 with the phosphate group in position 2 of kB region although it may occur in the position 3, contrary to X-ray data. The observed discrepancy may depend on minor differences in structure of the kB regions, in accord with the hypothesis according to which the position of the phosphate groups of DNA-protein contacts depends on the nucleotide sequence of the kB region [18].

Basing on the results obtained and on the literature data, the following, up-to-date optimal construction of DNA duplex capable of effective inhibiting the NF-kB transcription factor may be suggested:

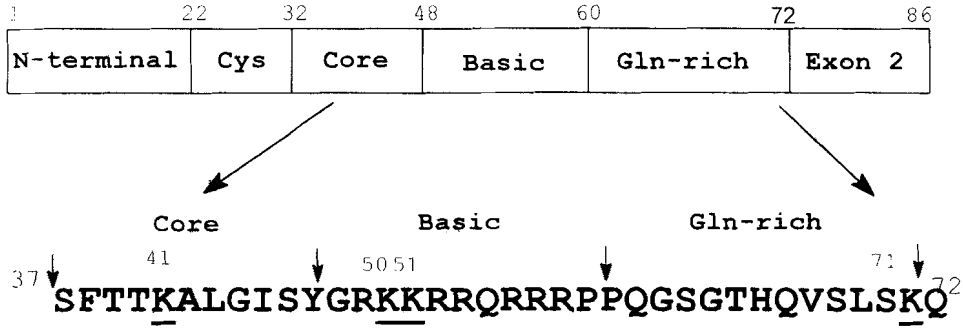


κB site

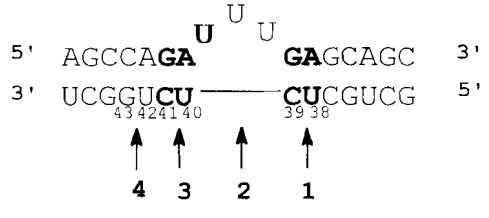
Such a DNA duplex must have a trisubstituted pyrophosphate group in the position 3 of the kB region because introduction of the active group in this position results in maximum efficacy of covalent binding to NF-kB p50 subunit. To ensure maximum thermostability and resistance to nucleases, the duplex complementary strands must be joined by a non-nucleotide bridge, e.g., by triethylene glycole, or by some other compounds, which may facilitate the penetration of such duplexes into cells. Synthesis of such DNA dumbbells containing TSP groups is described in [19].

Analogous experiments with dsRNA containing TSP groups was carried out in collaboration with Dr. M. Gait (Cambridge, UK) [6]. Chemical cross-linking of the HIV-1 Tat protein to synthetic models of the RNA recognition sequence TAR containing site-specific TSP linkage in place of a single phosphodiester was investigated. The structure of Tat protein and Tat peptide, as well as the structure of the HIV-1 TAR synthetic model duplex containing the U-rich bulge to which Tat binds is shown at the next scheme.

Tat protein



TAR



Arrows in the TAR model duplex denote the positions of incorporation of TSP. Method of synthesis of model TAR RNA duplexes was described earlier in this communication.

A TSP linkage was found to be reasonably stable to hydrolysis but cleaved under the action of ethylenediamine or lysine giving phosphoamidate adducts. A model HIV-1 TAR RNA duplex containing an activated TSP was able to bind to HIV-1 Tat protein with only 3-fold reduced affinity and to Tat peptide (residues 37-72) with identical affinity compared to that of an unmodified duplex. TSP incorporated at sites previously identified as being in close proximity to Tat protein were able to cross-link to Tat peptide (37-72) to form a covalent phosphoamidate conjugate. Endopeptidase cleavage followed by MALDI-TOF mass spectrophotometric analysis provided strong evidence that a TAR duplex containing TSP replacing the phosphate at 38-39 had reacted specifically with Lys 51 in the basic region of Tat peptide (37-72). The new chemical cross-linking method may be generally useful for identifying lysines in close proximity to phosphates in basic RNA-binding domains of proteins.

CONCLUSIONS

The new chemical cross-linking method may be generally useful for identifying lysines and histidines in close proximity to phosphates in basic DNA- or RNA-binding domains of proteins and then constituting a potential tool to control expression of disease-causing genes.

Since the covalent binding of the protein to DNA or RNA duplex with substituted pyrophosphate internucleotide linkage proceeds without external influence (e.g., UV, temperature, etc.), this decoy approach may be used in *in vivo* experiments and may find application in several branches of sense biotechnology.

ACKNOWLEDGEMENTS

This work was supported by grants N 97-04-48624 and 96-15-97707 from RFBR and in part by INTAS grant N 94/3810.

REFERENCES

1. Shabarova, Z. A. *Biochimie*, **1988**, ***36***, 1323-1334.

2. Shabarova, Z. A. *Physicochem. Biol. Rew. (SSR, Sect. D)*, **1984**, ***5***, 1-51.

3. Dolinnaya, N. G. and Shabarova, Z. A. *Izvestiya Acad. Nauk, ser. khim.*, **1996**, ***8***, 1889-1911 (English translation: *Russian Chem. Bull.*, **1996**, *45*, 1787-1809).

4. Kuznetsova, S. A., Clusel, C., Ugarta, E., Elias, I., Vasseur, M., Blumenfeld, M., and Shabarova, Z. A. *Nucl. Acids Res.*, **1996**, ***24***, 4783-4790.

5. Kozlov, I. A., Kubareva, E. A., Ivanovskaya, M. G., and Shabarova, Z. A. *Antisense and Nucl. Acids Drug Devel.*, **1997**, *7*, 279-289.

6. Naryshkin, N. A., Farrow, M. A., Ivanovskaya, M. G., Oretskaya, T. S., Shabarova, Z. A., and Gait, M. S. *Biochemistry*, **1997**, ***36***, 3496-3505.

7. Kuznetsova, S. A., Ivanovskaya, M. G., and Shabarova, Z. A. *Bioorg. Khymia*, **1990**, ***16***, 219-225.

8. Shabarova, Z. A. *Nucl. Acids Res., Symp. Ser.*, **1991**, ***24***, 99-103.

9. Ivanovskaya, M. G., Gottikh, M. B., and Shabarova, Z. A. *Nucleosides and Nucleotides*, **1987**, ***6***, 913-934.

10. Naryshkin, N. A., Ivanovskaya, M. G., Oretskaya, T. S., Volkov, E. M., Gait, M. J., and Shabarova, Z. A. *Bioorg. Khymiya*, **1996**, ***22***, 691-698.

11. Purmal, A. A., Shabarova, Z. A., and Gumport, R. I. *Nucl. Acids Res.*, **1992**, ***20***, 3713-3719.

12. Shabarova, Z. A., Sheflyan, G. Ya., Kuznetsova, S. A., Kubareva, E. A., Sysoev, O. N., and Ivanovskaya, M. G. *Bioorg. Khymiya*, **1994**, ***20***, 413-419.

13. Sheflyan, G. Ya., Kubareva, E. A., Volkov, E. M., Oretskaya, T. S., Gromova, E. S., and Shabarova, Z. A. *Gene*, **1995**, ***157***, 187-190.

14. Sheflyan, G. Ya., Kubareva, E. A., Kuznetsova, S. A., Karyagina, A. S., and Shabarova, Z. A. *FEBS Lett.*, **1996**, ***390***, 307-310.

15. Blumenfeld, M., Clusel, C., Ugarto, E., Kuznetsova, S., and Vasseur, M. *J. of Cell. Biochem.*, **1994**, ***186***, 42-45.

16. Chosh, G., Duyne, G. V., Chosh, S., and Sigler, P. B. *Nature*, **1995**, ***373***, 303-310.

17. Muller, C. W., Rey, F. A., Sedeoka, M., Verdine, G. L., and Harrison, S. C. *Nature*, **1995**, ***373***, 311-317.

18. Muller, C. W., Rey, F. A., Sedeoka, M.,Verdine, G. L., and Harrison, S. C., *Nature*, **1995**, ***373***, 224-227.

19. Kuznetsova, S. A., Blumenfeld, M., Vasseur, M., and Shabarova, Z. A. *Nucleosides and Nucleotides*, **1996**, ***15***, 1237-1251.