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Bridged Oligonucleotides as Molecular Probes for Investigation of Enzyme–Substrate Interaction and Allele-Specific Analysis of DNA

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Abstract—The efficiency of enzymatic conversion of DNA complexes containing non-nucleotide inserts has been studied. T4 DNA ligase and *Taq* DNA polymerase have been included in the study as examples of widely used DNA-dependent enzymes. A series of substrate DNA complexes have been formed using native oligonucleotides and bridged ones bearing non-nucleotide inserts based on phosphodiesters of di-, tetra-, or hexaethylene glycol, 1,5-pentanediol, 1,10-decanediol, and 3-hydroxy-2(hydroxymethyl)-tetrahydrofuran. The perturbation in DNA located far from the site of the enzyme action had almost no influence on the substrate properties of the complex, while insertion near this site significantly deteriorated them. The use of a series of modified duplexes allows one to locate the position of the enzyme-binding site on DNA substrate with the accuracy of 1-2 nucleotides. The presence of a non-nucleotide insert in the complex has been also shown to enhance the efficiency of single mismatch discrimination upon both template-directed ligation and extension of oligonucleotides.

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Oligonucleotides and their derivatives and analogs are universal molecular tools for investigation in biotechnology, molecular biology, and DNA diagnostics. Modified oligonucleotides having specified and improved properties as compared to the native precursors find increasing application for life sciences. For example, oligomers containing changed phosphodiester residues are more stable to degradation *in vivo* [1-3], which is important when designing biologically active compounds on the basis of nucleic acids (NAs), e.g. DNAzymes and antisense and antigene reagents [4, 5]. Different modifications can change hybridization properties of NA probes while preserving their sequence-specificity. There is a possibility to smooth hybridization properties of oligonucleotides, i.e. to align stability of G/C and A/T pairs [6-9], and even to enhance specificity in the formation of a hybridization complex [10]. Furthermore, the use of modified oligonucleotide primers promotes an increase in selectivity of allele-specific PCR [11-13].

Constructions based on modified oligonucleotides are used as tools for investigations of molecular-biological processes, e.g. NA–NA [14] or NA–protein [15, 16] interactions. Oligonucleotide derivatives can be employed for finer investigation of the mechanism of enzyme catalysis and for footprinting of binding sites between enzymes and NA substrates [17-19].

We previously studied complex formation of bridged oligonucleotides containing non-nucleotide inserts (bridges) between the native fragments. We evaluated thermodynamic parameters that allow accurate prediction of the influence of non-nucleotide inserts on the thermostability of complexes containing the bridged oligonucleotides [20, 21]. It was established that the extent of decrease in hybridization properties of the derivatives could be varied dependently on both the nature of the non-nucleotide insert and its location in the

Abbreviations: aa, amino acid residues; AU, activity units; NA, nucleic acid; prefix "d" in the designation of oligonucleotide sequences is omitted.

[#] Deceased.

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oligonucleotide chain. It was demonstrated that bridged oligomers could be used as the addressed part of antigene agents [22, 23] promoting specific action on NAs under physiological temperatures and as probes in hybridization analysis of DNA with participation of DNA-dependent enzymes [24, 25].

The influence of the location and nature of non-nucleotide inserts in substrate DNA complexes on the efficiency and selectivity of their enzymatic transformation was investigated with examples of widely used DNA-dependent enzymes—DNA ligase of T4 phage (T4 DNA ligase) and DNA polymerase of *Thermus aquaticus* (*Taq* DNA polymerase). It was shown that the use of series of substrate complexes based on bridged oligonucleotides containing a non-nucleotide insert in various positions allowed one to characterize the binding sites of enzymes on DNA. Unlike native DNA duplexes, the modified complexes were furthermore demonstrated to ensure enhanced efficiency in the discrimination of single nucleotide mismatches in DNA substrates in the course of enzymatic ligation and extension of oligonucleotides.

MATERIALS AND METHODS

We used T4 polynucleotide kinase, *Taq* DNA polymerase, T4 DNA ligase, dNTP, and ATP from Biosan (Russia).

Oligonucleotides were synthesized by the phosphoroamidite method on an ASM-800 DNA synthesizer (Biosset, Russia) using monomer synthons (Glen Research, USA). The modified synthons for the bridged oligodeoxyribonucleotides were synthesized from phosphodiesters of di-, tetra-, or hexaethylene glycol, 1,5-pentane- or 1,10-decanediol, and 3-hydroxy-2(hydroxymethyl)-tetrahydrofuran according to [26]. The resulting oligonucleotides were isolated and analyzed as described in [20].

Concentration of oligonucleotides was evaluated by measuring the optical density on a CARY 300 BioMelt spectrophotometer (Varian, Australia) and using the values of molar absorption coefficients (ε_{260}) for mono- and dinucleotides at 260 nm [27].

Oligonucleotide complexes were thermally denatured according to [28] at 26 μM sum concentration of the interacting oligonucleotides in the stoichiometric mixture in a buffer containing 10 mM sodium phosphate (pH 7.3), 1 M NaCl, and 1 mM EDTA. The melting temperatures of the complexes were calculated using the values of thermodynamic parameters obtained when analyzing the shapes of the corresponding melting curves.

5′-^{32}P-Labeled oligonucleotides were prepared using [γ-^{32}P]ATP (Institute of Chemical Biology and Fundamental Medicine, Siberian Division of Russian Academy of Sciences) [29].

Ligation. Native or modified dodecamer (10^{-5} M) and 5′-^{32}P-labeled octanucleotide ($5\cdot10^{-6}$ M) on 20-mer template (10^{-5} M) was ligated as described in [30] for 30 min at 25°C (if not specified) in the presence of T4 DNA ligase (40 AU) in buffer (10 μl) containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM ATP.

Extension. ^{32}P-labeled native or modified dodecanucleotide (10^{-6} M) on the 20-mer template (10^{-5} M) was extended in the presence of 1.5 AU of *Taq* DNA polymerase for 30 min at 50°C (if not specified) in buffer (10 μl) containing 10 mM Tris-HCl (pH 8.9), 50 mM KCl, 1.8 mM $MgCl_2$, 0.1% Tween-20, and $2\cdot10^{-4}$ M dNTP.

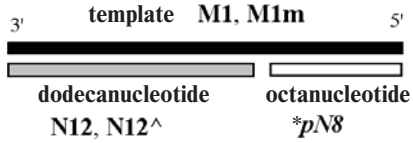
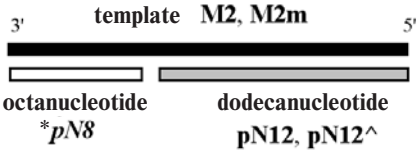
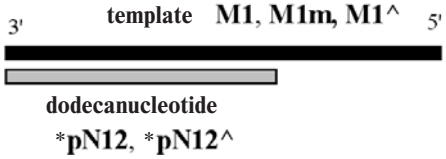
The products of the enzyme reactions were analyzed by gel electrophoresis in denaturing 20% polyacrylamide gel. Chemically synthesized oligonucleotides were used as length markers. The gel was radioautographed onto Curix X-ray film (AGFA, Belgium), which was then scanned on a Umax instrument (PowerLook 1000, Germany). The results were evaluated using the GelPro Analyzer 4.0 program package (Media Cybernetics, USA); the yields of reaction products were estimated taking into account the relative distribution of the integral optical density (IOD) in the lanes. The discrimination factor was calculated as the yields ratio of full-size products of the enzyme reaction in cases of the right complex and the complex containing the nucleotide polymorphism.

RESULTS

Description of model systems. We used three model systems (Table 1): models L1 and L2 for the reactions of oligonucleotide ligation with T4 DNA ligase and model E for the reaction of the extension of an oligonucleotide primer with *Taq* DNA polymerase. Systems L1 and L2 differed by the relative disposition of the binding sites for the ligated dodecanucleotide and octanucleotide. In system L1, native (N12) or modified (N12^) dodecanucleotides were used as OH components and octanucleotide *pN8* – as the phosphate-containing component in the ligation reaction. Unlike that, octanucleotide *pN8* was used as OH component and dodecanucleotides pN12 or pN12^ – as donors of the 5′-terminal phosphate in system L2 (Table 1). System E was employed for studying the extension of dodecanucleotides pN12 or pN12^ with *Taq* DNA polymerase. The enzyme reactions were monitored using ^{32}P-label in one of the components of the model system.

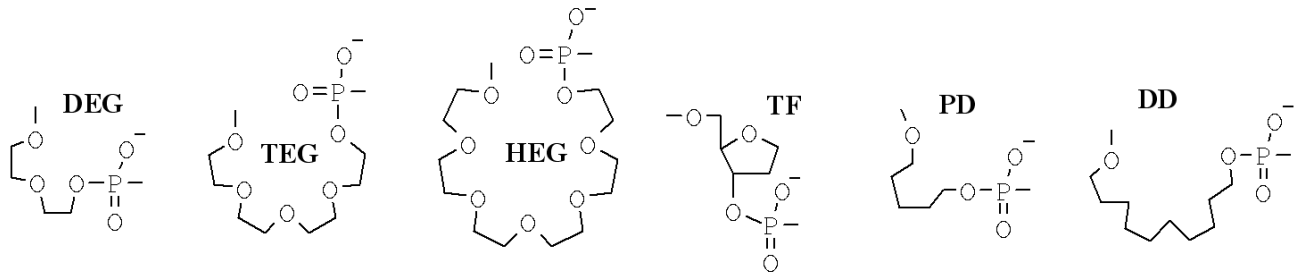
Depending on the model, the studied complexes were formed from native octanucleotide *pN8*, native or modified dodeca- (N12, pN12, N12^, or pN12^), or eicosanucleotides (M1, M2, M1m, M1^, M2, or M2m) (Table 2). The sugar-phosphate backbone was modified using non-nucleotide inserts (^) based on phosphodi-

Table 1. Structure and composition of model systems

Model		Structure	Composition
Ligation	L1		**M1/N12 + *pN8*** **M1/N12^ + *pN8*** **M1m/N12 + *pN8*** **M1m/N12^ + *pN8***
	L2		**M2/*pN8* + pN12** **M2/*pN8* + pN12^** **M2m/*pN8* + pN12** **M2m/*pN8* + pN12^**
Extension	E		**M1/pN12** **M1/pN12^** **M1m/pN12** **M1m/pN12^** **M1^/pN12**

* ^{32}P-labeled component.

^ Here and in Table 2, non-nucleotide inserts:



esters of di- (DEG), tetra- (TEG), or hexaethylene glycol (HEG), 3-hydroxy-2(hydroxymethyl)-tetrahydrofuran (TF), 1,5-pentane- (PD), or 1,10-decanediol (DD). The DEG, TEG, HEG, TF, PD, or DD residues were introduced in oligomer N6^6. Oligomer N6$^{(\wedge)5}$6 contained five successive DEG residues. The inserts were introduced in various positions of the oligonucleotide chain. The DEG residue was distanced by two (N2^10) or ten (N10^2) nucleotides from the 5′-end of dodecanucleotides N12^ and by four (M4^16) or 18 (M18^2) nucleotides from the 5′-end of eicosanucleotides. In all cases the insertions led to the formation of a non-nucleotide loop in the duplex.

Templates M1, M2, and M1^ contained the completely complementary sites for dodecanucleotides N12 or N12^ and octanucleotide *pN8*. The binding site for octanucleotide *pN8* (italic in Table 2) was at the 3′-end of templates M1, M1m, and M1^ (models L1 and E) and at the 5′-end of templates M2 and M2m (model L2). The non-nucleotide diethylene glycol residue (DEG) was inserted into template (M1^) both in the dodecanucleotide binding site (templates M18^2-to-M10^10) and outside this site in the positions of the first (templates M8^12) and fifth (templates M4^16) nucleotides in the overlapping single-stranded fragment of the template strand (Table 2). Templates M1m and M2m differed from templates M1 and M2 by single-nucleotide substitutions in position "m" relatively the 3′-end of the OH component or primer (in ligation or extension reactions, respectively). The "m" value has the sign "–" or "+" if the nucleotide substitution is located in the position corresponding to the direction 3′→5′ or 5′→3′ from the 3′-end of the transformed component, respectively (Table 2).

The stability of the substrate complexes formed from the native and bridged oligonucleotides were studied by the thermal denaturation method. The experimental results confirm that the non-nucleotide inserts in an oligonucleotide weaken its ability for complex formation (Fig. 1, a and b). The melting temperatures of complexes of the bridged oligonucleotides N12^ are lower than that of the control complex of dodecamer N12 (56.3°C) by 3-12.5°C. The maximal destabilization (–12.5°C) of the duplex formed by modified dodecanucleotide N12^ with

Table 2. Sequences of native oligonucleotides (*pN8*, N12, M1, M1m, M2, and M2m) and bridged oligonucleotides containing non-nucleotide inserts (N12^ and M1^)

Designation		Sequence
pN8		5'-*pGCATCAAG*
(p)N12		5'-(p)CAGCTCCAGGCA
	(p)N2^10	5'-(p)CA^GCTCCAGGCA
	(p)N3^9	5'-(p)CAG^CTCCAGGCA
	(p)N4^9	5'-(p)CAGC^TCCAGGCA
	(p)N5^7	5'-(p)CAGCT^CCAGGCA
(p)N12^	**(p)N6^6**	5'-(p)CAGCTC^CAGGCA
	(p)N7^5	5'-(p)CAGCTCC^AGGCA
	(p)N8^4	5'-(p)CAGCTCCA^GGCA
	(p)N9^3	5'-(p)CAGCTCCAG^GCA
	(p)N10^2	5'-(p)CAGCTCCAGG^CA
	(p)N6$^{(^)5}$6	5'-(p)CAGCTC^^^^^CAGGCA
M1		5'-*CTTGATGC***TGCCTGGAGCTG**
	M1–1	5'-*CTTGATGC***c**GCCTGGAGCTG**
	M1–2	5'-*CTTGATGC***Ta**CCTGGAGCTG**
M1m	**M1–3**	5'-*CTTGATGC***TGtCTGGAGCTG**
	M1–4	5'-*CTTGATGC***TGCtTGGAGCTG**
	M1–5	5'-*CTTGATGC***TGCCcGGAGCTG**
	M1–6	5'-*CTTGATGC***TGCCTaGAGCTG**
	M4^16	5'-*CTTG^ATGC***TGCCTGGAGCTG**
	M8^12	5'-*CTTGATGC^***TGCCTGGAGCTG**
	M10^10	5'-*CTTGATGC***TG^CCTGGAGCTG**
M1^	**M12^8**	5'-*CTTGATGC***TGCC^TGGAGCTG**
	M14^6	5'-*CTTGATGC***TGCCTG^GAGCTG**
	M16^4	5'-*CTTGATGC***TGCCTGGA^GCTG**
	M18^2	5'-*CTTGATGC***TGCCTGGAGC^TG**
M2		5'-**TGCCTGGAGCTG***CTTGATGC*
	M2–1	5'-**TGCCTGGAGCTG***t**TTGATGC*
	M2–4	5'-**TGCCTGGAGCTG***CTTt**ATGC*
M2m	**M2+4**	5'-**TGCCTGGAtCTG***CTTGATGC*
	M2+5	5'-**TGCCTGGtGCTG***CTTGATGC*
	M2+9	5'-**TGCtTGGAGCTG***CTTGATGC*

Note: (p), the presence of the terminal phosphate. Octanucleotide *pN8* and its binding site with template M are italicized; the binding site for dodecanucleotide N12 is bold. The single-nucleotide discrepancies in the dodecanucleotide N12 and template M binding site are underlined.

DNA template is observed when the insert is introduced in the center of oligomer N6^6. It should be noted that the tandem complexes used as substrates for DNA ligase are more stable as compared to the complexes between the template and the individual components (N12 and *pN8*) (data not shown). This is caused by cooperative interactions in the junction of neighboring helix regions in DNA [31, 32]. The thermal stability values for complexes formed by the bridged oligonucleotides were calculated using our previously developed computer algorithm [20, 21]. The results show that all of the studied complexes have a reasonable stability, which is characterized by melting temperature (T_m) close to or exceeding the temperature used in the experiment.

The presented series of the model systems and the variety of their components allows one to methodically study the influence of the nature of the non-nucleotide inserts and their position in the structure of the substrate complex on the efficiency of the enzymatic ligation and extension in the perfect and imperfect duplexes of the bridged oligonucleotides.

Influence of non-nucleotide inserts in substrate complexes on efficiency of their transformations catalyzed by DNA-dependent enzymes. The ability of complexes formed by the bridged oligonucleotides to be substrates for DNA-dependent enzymes was studied with examples of T4 DNA ligase and *Taq* DNA polymerase widely used in various investigations and in practice.

Ligation of perfect DNA complexes by T4 DNA ligase. Model systems L1(M1/N12^ + *pN8*) and L2(M2/*pN8* + pN12^) (Tables 1 and 2) were used to study the influence of the position of the non-nucleotide inserts in the modified complexes on the efficiency of their ligation. Ligation was carried out under the conditions of efficient complex formation at the temperature (25°C) optimal for the enzyme [33]. The efficiency of the enzyme reaction decreases as the non-nucleotide insert is moved closer and closer to the ligation site (Fig. 1). In complexes of the L1 series containing the insert in the OH-component, the efficiency of the formation of the modified product for oligomers N2^10-to-N6^N6 (i.e. when the non-nucleotide insert is distant from the single-stranded nick by six and more nucleotides) is comparable with that for native oligonucleotide N12 (Fig. 1). The decrease in extent of formation of the full-length ligation product is observed when the non-nucleotide insert separates five or less nucleotide units from the 3′-OH ligation end of dodecanucleotide N12^. In this case, we observe accumulation of an additional product corresponding to the 5′-adenylated form (*AppN8*) of the phosphate-containing component *pN8* [33]. In L2 complexes, when the insert is localized in the P-component, pronounced inhibition effect of the insert on the enzyme ligation is observed with some longer distance from the ligation site (seven or fewer nucleotides) (Fig. 1).

The results show that the influence of the position of the non-nucleotide insert in OH- and P-components on

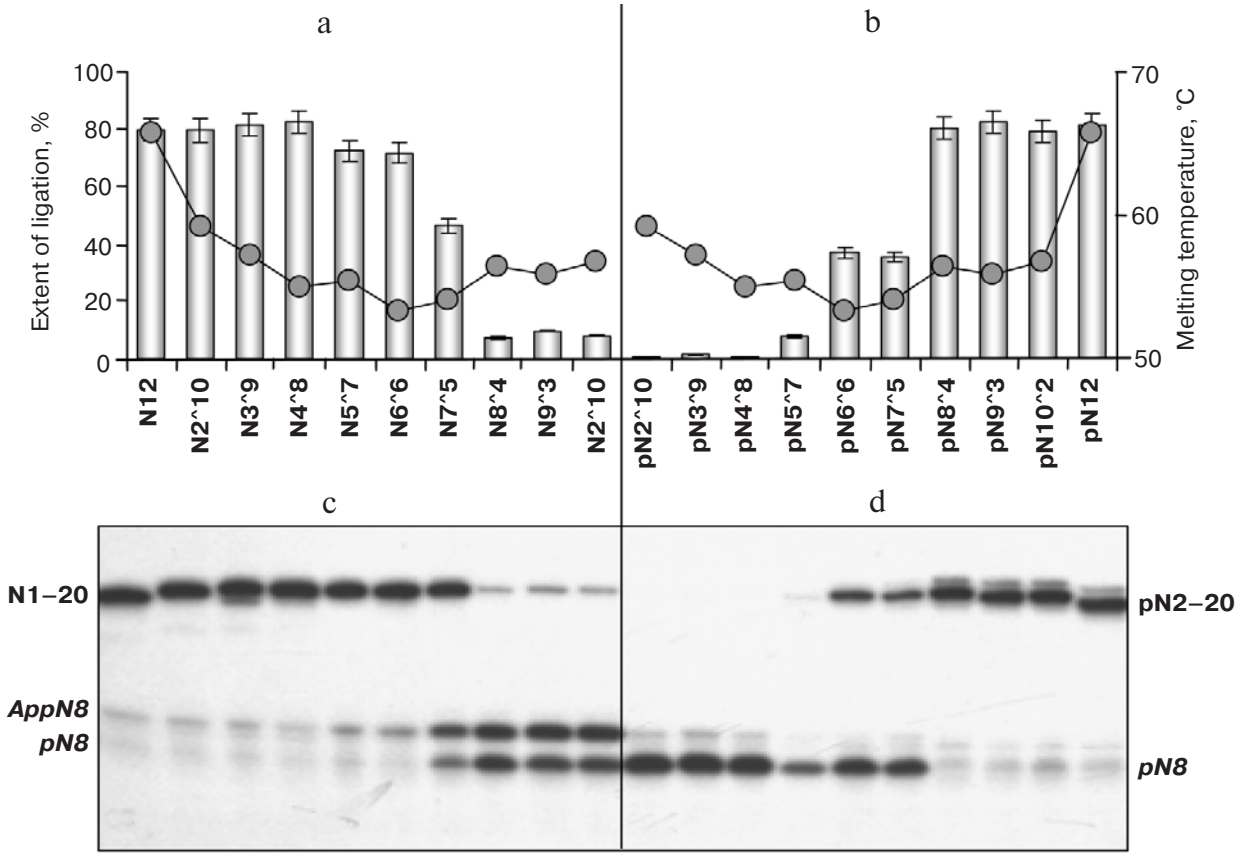


Fig. 1. T4 DNA ligation of native and modified complexes L1(M1/N12, N12^ + *pN8*) and L2(M2/*pN8* + pN12, pN12^) differing from each other in the position of the non-nucleotide insert relative to the single-stranded nick, which are determined by the sequences of dodecanucleotides N12, N12^. a, b) Extent of oligonucleotide ligation in complexes L1 and L2 (columns) and melting temperatures of complexes formed by the corresponding dodecanucleotide (N12, N12^) and template 5′-TGCCTGGACGTG-3′ (circles). c, d) Results of electrophoretic analysis in 20% denaturing polyacrylamide gel (8 M urea) of products of ligation in model complexes L1 and L2. *pN8*, *AppN8*, N1-20, and pN2-20 are positions of the starting labeled octanucleotides and ligation products. See "Materials and Methods" for the ligation conditions.

the enzyme ligation efficiency is asymmetrical relative to the ligation site.

Ligation of native and bridged oligonucleotides in imperfect DNA complexes. The efficiency in discrimination of single-nucleotide mismatches between sequences of DNA template and modified oligonucleotides was studied using systems L1(M1m/N12^ + *pN8*) and L2(M2m/*pN8* + pN12^) (Tables 1 and 2). Oligomers N6^6 (for L1) and pN8^4 (for L2) were used as bridged oligonucleotides because the ligation efficiency in the corresponding perfect complexes L1(M1/N6^6 + *pN8*) and L2(M2/*pN8* + pN8^4) almost coincides with that for the control complexes L1(M1/N12 + *pN8*) and L2(M2/*pN8* + pN12) (Fig. 1). Oligonucleotides M1m and M2m containing single-nucleotide substitutions were used as templates (Table 2).

It was shown using complexes L1(M1m/N6^6 + *pN8*) (m = –1 ... –6) that all of the nucleotide substitutions in templates M1-1-to-M1-6 are efficiently discriminated, i.e. the ligation yield decreases to 10% (Fig. 2a). The shift of the mismatch from the single-stranded nick up to position –6 (template M1-6) slightly enhances the observed efficiency of ligation of the imperfect complexes. At the same time, the presence of the mismatches in control duplex L1(M1m/N12 + *pN8*) decreases the ligation yield only when they are located near the single-stranded nick in positions –1 (template M1-1) and –2 (template M1-2) (Fig. 2a). As the mismatch is shifted from the single-stranded nick, the efficiency of ligation of the imperfect complex is recovered step-by-step up to 68 and 74% in complexes L1(M1-5/N12 + *pN8*) and L1(M1-6/N12 + *pN8*), respectively, which is comparable with ligation in control system L1(M1/N12 + *pN8*) (84%) (Fig. 2a).

DNA ligases are characterized by low efficiency in discrimination of mismatches localized in the phosphate-containing component of a substrate [34, 35]. Unlike this, we observed decrease in the extent of ligation of the bridged oligonucleotide on the corresponding wrong templates in system L2(M2m/*pN8* + pN8^4) (m = +4, +5). These point substitutions are not discriminated

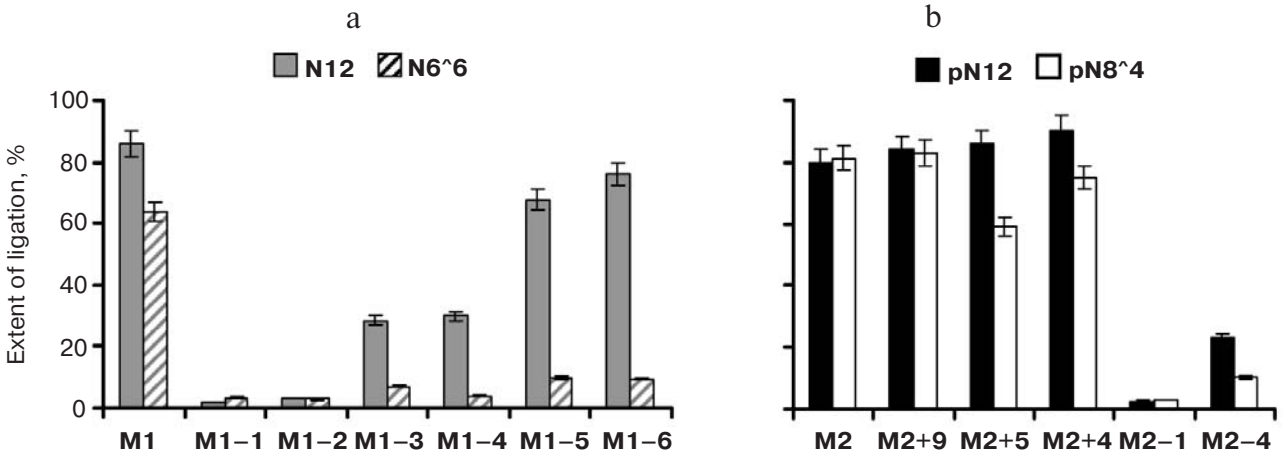


Fig. 2. Dependence of the yield of the full-size ligation product in control and modified complexes L1(M1, M1m/N12, N6^6 + *pN8*) (a) and L2(M2, M2m/*pN8* + pN12, pN8^4) (b) on the distance of the single-nucleotide substitution relatively the single-stranded nick, which is determined by the sequences of templates M1m and M2m. See "Materials and Methods" for ligation conditions.

when the native oligonucleotides are ligated in system L2(M2m/*pN8* + pN12). The mismatch, most distant from the ligation site (template M2 + 9), does not reduce the efficiency of transformation of either pN12 or pN8^4 as compared to perfect complex (Fig. 2b).

Influence of nature of non-nucleotide insert on ligation of bridged oligonucleotides. We studied the influence of the nature and length of non-nucleotide inserts on the ability of modified complexes to be substrates for T4 DNA ligase. To this end, we used perfect and imperfect complexes L1(M1 or M1-6/N6^6 + *pN8*; M1 or M1-6/N6$^{(\wedge)5}$6 + *pN8*). The bridged oligonucleotides containing any of the residues (DEG, TF, PD, TEG, DD, or HEG) in N6^6 or $(DEG)_5$ in N6$^{(\wedge)5}$6 (Tables 1 and 2) were under consideration. Oligomer M1-6 was used as the imperfect template resulting in mismatch in the sixth position from the site of ligation in the 3′→5′ direction.

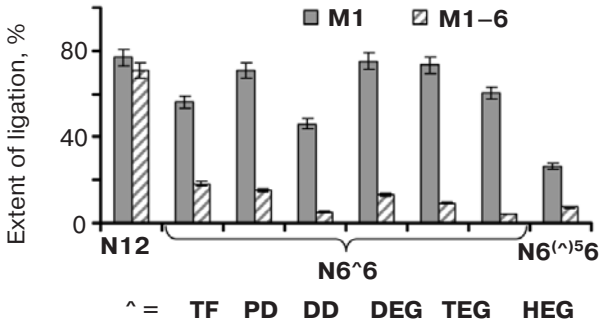


Fig. 3. Extent of ligation in complexes L1(M1, M1-6/N12, N6^6, N6$^{(\wedge)5}$6 + *pN8*) of native (N12) and modified (N6^6 or N6$^{(\wedge)5}$6) dodecamers with octanucleotide *pN8* on right template M1 and template M1-6 containing single-nucleotide substitution. Inserts in dodecamers differ by nature and length of the non-nucleotide insert (^). See "Materials and Methods" for ligation conditions.

The nature of the insert in the OH component shifted from the ligation site by six nucleotides inhibits the observed enzyme efficiency (Fig. 3). The most noticeable decrease in the yield of the ligation product is promoted by the looped inserts based on non-flexible cyclic tetrahydrofuran (TF), hydrophobic decanediol (DD), the bulkiest residue of hexaethylene glycol (HEG), and by insert consisting of five successive residues of diethylene glycol phosphate $(DEG)_5$. The inserts of either nature and length in the imperfect complex L1(M1-6/N6^6 + *pN8*) significantly enhance the ability of DNA ligase to discriminate mismatches remote from the ligation site (Fig. 3).

Extension of bridged oligonucleotides in perfect DNA-complexes with use of Taq DNA polymerase. The influence of non-nucleotide inserts in the oligonucleotide primer on polymerization efficiency was studied using model system E(M1/pN12^) (Table 1). The diethylene glycol residue was inserted in various positions of the elongated dodecanucleotide. It was shifted by 2 to 10 nucleotides from the 3′-end of pN12^ (Table 2).

Both the control and the bridged dodecanucleotides can be elongated to the full-size product in perfect complexes E(M1/pN12) and E(M1/pN12^) (Fig. 4a). However, the efficiency of the extension of the bridged oligonucleotides appeared to significantly depend on the position of the disturbance in the primer (Fig. 4a). Oligomers pN2^10-to-pN6^6 containing the insert shifted by 6-10 nucleotides from the 3′-end are elongated to the full-size product with nearly the same efficiency (70-80%) as the control dodecanucleotide pN12 (86%). If the insert is shifted by 2-5 nucleotides from the 3′-end of oligomer (pN7^5-to-pN10^2), the efficiency of extension noticeable decreases.

Bridged oligonucleotides as templates in the polymerase reaction. The effects caused by the non-nucleotide inserts in the template M1^ were studied in E(M1^/pN12) com-

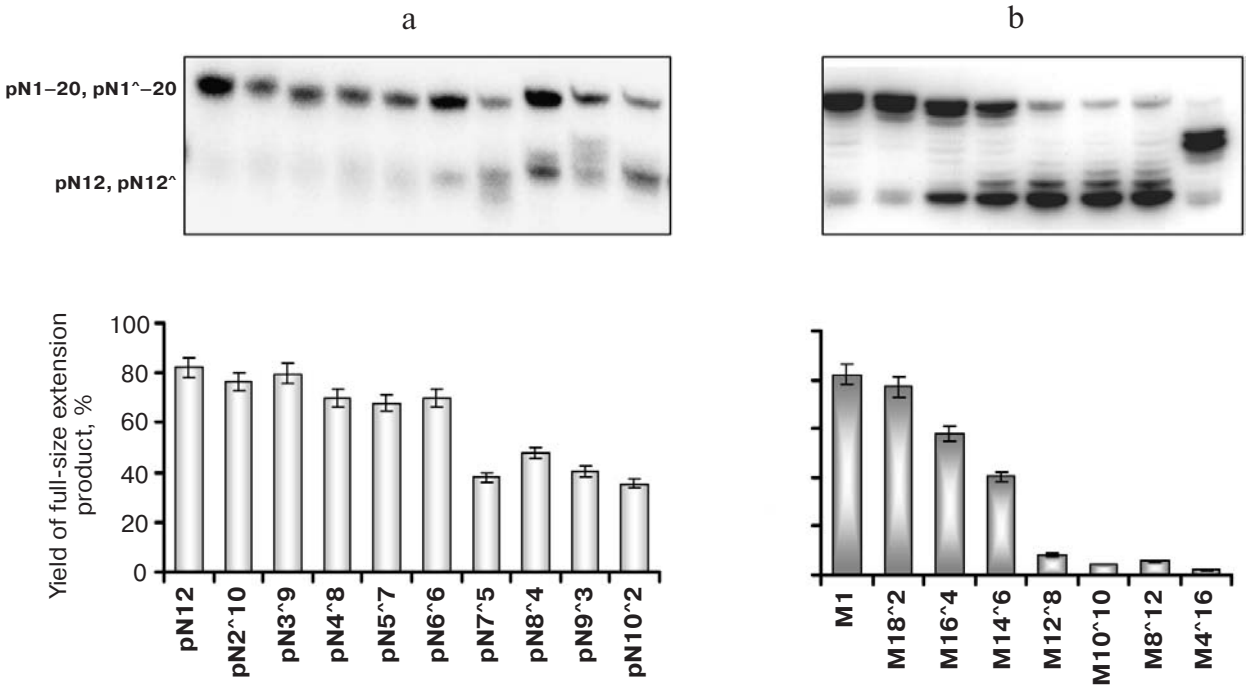


Fig. 4. *Taq* DNA polymerase extension of dodecanucleotides in native and modified complexes of the E series. Results of electrophoretic analysis in 20% denaturing polyacrylamide gel (8 M urea) of extension products and yield of full-size product in control and modified complexes E(M1/pN12) and E(M1/pN12^) containing the non-nucleotide insert in various positions of dodecanucleotide pN12^ (a) and in control and modified complexes E(M1/pN12) and E(M1^/pN12) containing the non-nucleotide insert in various positions of template M1^ (b). pN12, pN12^, pN1-20, and pN1^-20 are positions of starting labeled dodecanucleotides and extension products. See "Materials and Methods" for extension conditions.

plexes using native dodecanucleotide primer pN12 (Table 1). It is shown that only the modified duplex E(M18^2/pN12) containing the most distant insert from the elongated 3′-end of dodecanucleotide pN12 provides nearly the same yield of the product as does native duplex E(M1/pN12) (Fig. 4b). Migration of the non-nucleotide insert closer to the 5′-end of the template (M16^4 and M14^6) and thereby to the 3′-terminal region of primer pN12 leads to decline in the yield of the full-size product to 51 and 36%, respectively. Further shift of the insert (templates M12^8 and M10^10) results in significant inhibition of formation of the full-size product. It should be noted that in these cases the extension of pN12 by one nucleotide takes place (up to 15%). The presence of diethylene glycol residue outside duplex E(M8^12/pN12), in the first position of the overhanging single-stranded region, leads to termination of chain growth after the incorporation of one nucleotide. In case of template M4^16, dodecanucleotide pN12 is completed up to the modification site, i.e. by four complementary nucleotides and by one more in addition. It has been shown in a special investigation that *Taq* DNA polymerase can incorporate all possible nucleotides in the position opposite the diethylene glycol residue, however, with different efficiency, which follows the trend AMP ≈ GMP > TMP ⩾ CMP (data not shown).

It is important to note that decrease in the reaction temperature from 50 to 37°C does not change the character of the influence of the insert position in the substrate complexes on efficiency of primer extension (data not shown). This means that the observed effects are not the result of the difference in the stability of the modified complexes.

Extension of bridged oligonucleotides in imperfect complexes. We used model system E(M1m/pN12^) (Tables 1 and 2) to study the selectivity of *Taq* DNA polymerase extension of bridged oligonucleotides pN12^ in complexes with templates M1m. Oligomers pN12^ contained the DEG insert in a distance of 2-10 nucleotides from the 3′-end. The selectivity of the reaction was evaluated by comparing the extension efficiency for the perfect and imperfect complexes containing the single mismatches in various positions of the duplex (Fig. 5a). The selectivity of the primer extension in control complex E(M1m/pN12) is low. The most significant decrease in yield of full-size product is caused by mismatch in the position opposite the 3′-end of the dodecamer (template M1-1; Fig. 5b). The efficiency of the extension of native pN12 in templates M1-2-to-M1-6 becomes comparable to that in the perfect complex E(M1/pN12).

The efficiency of the extension of imperfect complexes E(M1m/pN12^) containing the non-nucleotide

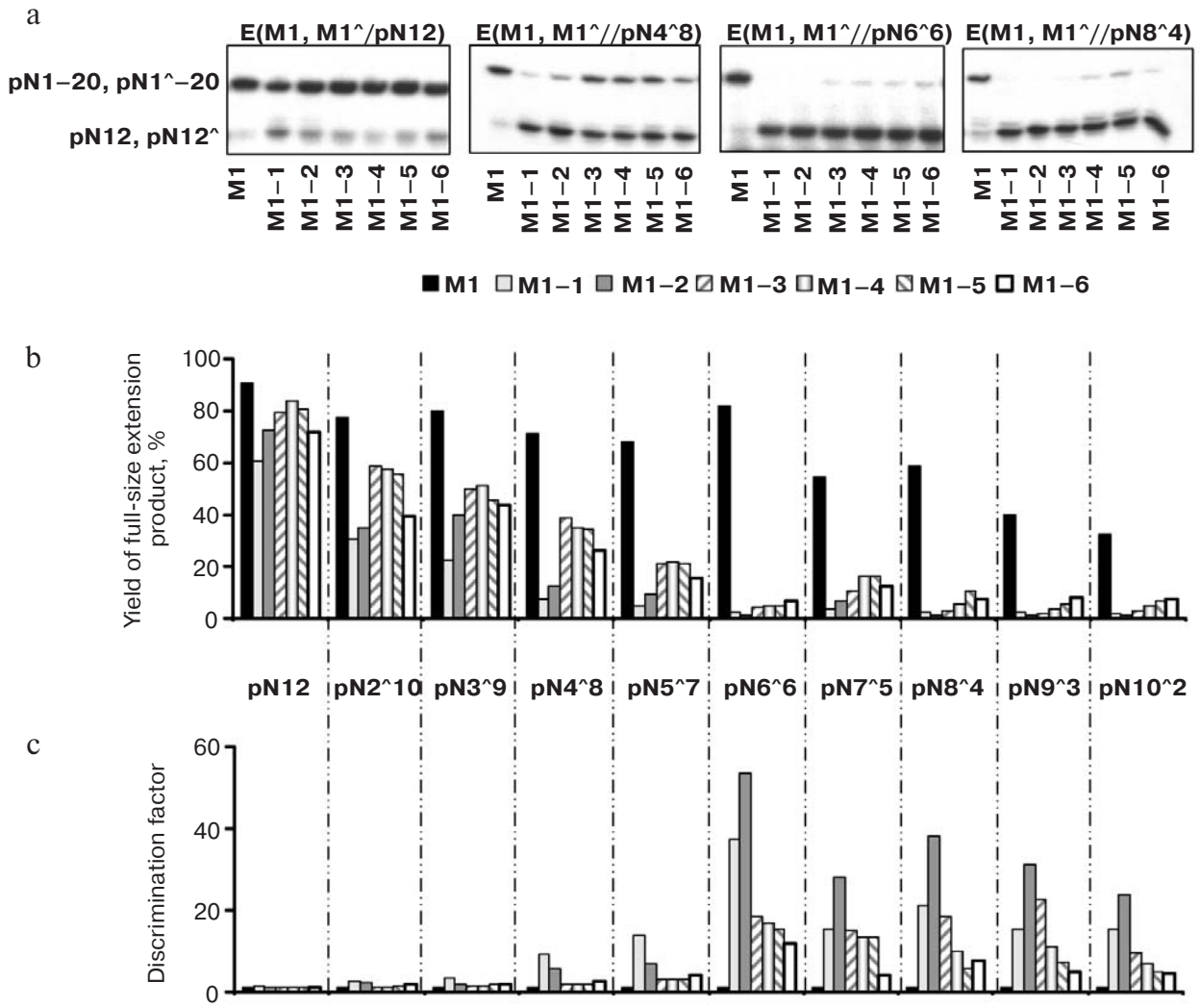


Fig. 5. Dependence of yield of full-size product of *Taq* DNA polymerase extension of native (pN12) and modified (pN12^) dodecanucleotides in imperfect E complexes on distance of non-nucleotide insert. a) Results of electrophoretic analysis in 20% denaturing polyacrylamide gel (8 M urea) of extension products of dodecanucleotides pN12, pN4^8, pN6^6, and pN8^4 in the presence of the right template (M1) and templates containing single-nucleotide discrepancies (M1m). pN12, pN12^, pN1-20, and pN1^-20 are positions of starting labeled dodecanucleotides and extension products. b) Yield of full-size extension products of pN12 and bridged pN12^ in the presence of templates M1 and M1m. c) Discrimination factors. See "Materials and Methods" for extension conditions.

loop appears to be always lower than that of the corresponding perfect complexes E(M1/pN12^) (Fig. 5, a and b). The position of the non-nucleotide insert in the bridged oligomers significantly influences the selectivity of their extension. In general, the more distant the insert from the 3′-end of the modified oligonucleotide, the less impact it causes on the extension efficiency in the imperfect complex. The bridged oligonucleotides can be divided in two groups based on the character of the observed effects (Fig. 5, b and c). The first group consists of oligomers pN2^10-to-pN5^7, which are characterized by the high efficiency of their extension in the perfect complex (~70%) in combination with a relatively low sensitivity to the single-nucleotide polymorphism in the template. In the second group (pN6^6-to-pN10^2), rather reduced efficiency of the reaction of the perfect complex (Fig. 5b) is combined with a high mismatch discrimination factor (Fig. 5c). On average, bridged oligonucleotide pN6^6 bearing the insert in the middle of the chain is the most efficient for the discrimination of mismatches in various positions of the imperfect complexes, with insignificant decreasing of the extension efficiency. The decrease of the reaction temperature from 50 to 37°C provides the enhancement of the association between oligomers and the DNA template; on the other hand, it weakens the efficiency of *Taq* DNA polymerase. This leads to a decrease in the efficiency of the oligomer extension for all studied templates but does not change the character of the mismatch discrimination in the corresponding imperfect complexes (data not shown).

DISCUSSION

Non-nucleotide inserts in the sugar-phosphate backbone disturb the regularity of the oligomer chain. The insert in the bridged oligonucleotide forms the loop in a certain position of the duplex structure. The change of the nature or the size of the insert allows regulating the extent of the local disturbance in the DNA duplex structure and thereby influencing the efficiency of complex formation [20]. The previously obtained uniform thermodynamic parameters of the DNA/DNA complex formation allow one to preliminarily evaluate their stability [21]. However, the substrate properties of the bridged oligonucleotides in the reactions of DNA-dependent enzymes are determined not only and not so much by the thermostability of their complexes but mostly by the type, the size, and the position of the non-nucleotide inserts. Systematic investigations are required in order to determine the characteristics of the reactions of the bridged oligonucleotides catalyzed by DNA-dependent enzymes.

Characteristics of enzyme transformations of perfect complexes formed by bridged oligonucleotides. The non-nucleotide insert causes a local disturbance in the DNA double helix, with the B-form of the whole duplex being preserved [20]. Investigation of the looped DNA complexes by NMR spectroscopy shows that an insert induces a bend in the modified region of the double helix, which increases with increasing insert size [36, 37]. The enhanced accessibility of the base pairs in this site for attack by water leads to the acceleration of the dissociation rate of the modified double helix as compared to the native one [20]. Thus, the non-nucleotide insert in complexes of the bridged oligonucleotides causes their destabilization, with the extent of this effect being determined by the nature and the length of the insert [20].

The cumulative physicochemical data concerning the structure of DNA complexes containing non-nucleotide inserts-loops allow one to assume that such duplexes can be substrates in the enzyme reactions and that the influence of an insert should be determined by its localization in the DNA substrate.

We used several series of model complexes (Tables 1 and 2) to study the influence of various non-nucleotide inserts (oligoethylene glycol and oligomethylenediol residues) in DNA complexes on the efficiency of their conversion in the presence of T4 DNA ligase and *Taq* DNA polymerase.

The results demonstrate that this influence is caused by modification of the enzyme-binding site. A general relationship is observed for both enzymes, namely, approach of an insert to the site of the enzyme substrate conversion decreases reaction yield (Figs. 1 and 4). The size and the nature of the non-nucleotide residue play, apparently, a secondary role changing only the extent of the effect of the insertion in a certain position of the substrate complex (Fig. 3).

The insert position relative the single-stranded nick impacts the efficiency of T4 DNA ligation of the bridged oligonucleotides (Fig. 1). In complexes of L1 series, the diethylene glycol residue significantly influences ligation when being remote from the 3′-end of the OH component by not more than five nucleotides. In case of the modified P component in complexes of L2 series, a significant effect is revealed at some longer distance (seven nucleotides from the ligation site). Modifications of the substrate complex inside the specified boundaries lead to almost complete inhibition of the enzyme reaction. It should be noted that the decrease in the yield of the ligation product in complexes L1 in contrast to that in complexes L2 is associated with the accumulation of 5′-adenylated octanucleotide P component (*AppN8* in Fig. 1a). This is the evidence of the fact that T4 DNA ligase recognizes substrate complexes containing a modified OH component, activates a 5′-phosphate component, but cannot form a new phosphodiester bond. The low efficiency in ligation of L2 complexes is, apparently, caused by troubles at the enzyme–substrate binding stage because even the activation of the P component does not occur.

The modified substrate regions, which deteriorate the substrate properties of tandem complexes, have various lengths. This reflects the asymmetry in the disposition of the interaction zone between T4 DNA ligase and DNA substrate relative to the ligation site (from –5 to +7). Analogous features of the enzyme–substrate interaction were revealed for a number of DNA ligases [38, 39]. It is important to note that the size of the enzyme-binding site fixed in this work is functionally significant for the formation of the active enzyme–substrate complex. In contrast to that, the radical or nuclease footprinting evaluates the length of the DNA region covered by the enzyme upon binding. Footprinting with exonuclease III shows that the smallest DNA ligase from *Chlorella virus* (34 kDa, 298 aa) can cover 19-21 nucleotides in the DNA substrate [39], while for T7 DNA ligase (41 kDa, 359 aa) having a little less size than T4 DNA ligase (55 kDa, 487 aa), the binding site is 12-14 nucleotides [38], which is comparable with the results presented here and in [40].

The experiments with *Taq* DNA polymerase and model complexes of E series (Fig. 4) yields more definitive data demonstrating the adequacy of the proposed approach to the determination of the enzyme binding site with the use of the set of modified substrates. We showed that the DEG insert in the primer reveals its negative effect at a distance of less than five nucleotides from the extended 3′-end. The presence of the same insert in the template becomes pronounced when eight nucleotides separate it from the enzyme reaction site. These results are in complete agreement with the data of X-ray analysis of the complex DNA substrate/*Taq* DNA polymerase [41, 42], which show specific tight interaction between amino acid residues of the enzyme and 8-9 and 5-7

nucleotides in the template and primer strands, respectively.

It is important to note that the presence of a non-nucleotide insert in the template strand is more crucial than a disturbance in the primer strand (Fig. 4). The presence of the inserts in M10^10 and M12^8 near the conversion site (positions –2 and –4, respectively) leads to almost complete inhibition of the enzyme reaction. The non-nucleotide insert in the primer strand has not so significant consequences independently of its position. The modification in the single-stranded region of DNA template, where the primer extension occurs, is a "stop" signal for DNA polymerase, which is in agreement with work [43]. The enzyme introduces one, predominantly purine, nucleotide opposite the non-nucleotide unit followed by the break of the extension, which also conforms to the literature data [44]. Nevertheless, the insignificant amount of the full-size product along with products of the incomplete reaction are formed in case of templates M10^10, M8^12, and M4^16. This means that DNA polymerase can overcome the injured site, although with a low efficiency.

Additional information about the enzyme–substrate interaction can, obviously, be obtained when studying the dependence of the substrate properties of modified complexes on the nature of an insert that is located in the proximity to the enzyme-binding site. In case of T4 DNA ligase, we showed that the loops formed from the non-flexible cyclic TF, hydrophobic DD, and bulky HEG- and $(DEG)_5$ residues noticeably decrease the ligation efficiency when they are distant from the ligation site by six nucleotides. The inserts formed from phosphodiesters of di- and tetraethylene glycol and pentanediol in this position have no significant influence on the enzyme reaction. One can assume that above effects observed at temperatures of the efficient complex formation for all of the modified substrates can be caused only by peculiarities of the spatial organization in the modified enzyme–substrate complexes and/or by the differences in conformation dynamics. The decreased efficiency of the enzyme conversion of the modified perfect complexes can be explained by the difficulty of the formation of the contacts necessary to the organization of the full-value complex between DNA-substrate and enzyme.

Thus, we suggested a new approach to the precise evaluation of the region in DNA substrate (1-2 nucleotides) interacting with an enzyme. This approach is based on the use of DNA complexes formed from bridged oligonucleotides while varying the position of the non-nucleotide insert relative to the enzyme conversion site. The method was tested with an example of two enzymes. The advantage of using the bridged oligonucleotides is that we can indirectly estimate the extent of the interaction between the enzyme and nucleotides flanking the non-nucleotide insert. The proposed method can be additionally applied to the evaluation of the spatial characteristics of the complex outside the DNA helix by means of changing the volume characteristics of the loops. It is also possible to model the conformation dynamics of the loops by changing the nature of the non-nucleotide insert. The efficiency of the enzyme conversion of the modified perfect complexes decreases due to damaging contacts that are necessary to the formation of the full-value enzyme–substrate complex. At the same time, the insertion disturbs the regularity of the DNA structure. As a result, the duplex region covered by the enzyme can lose its ability for the transformation from the B- to A-like form. These changes in the substrate structure in the proximity of the enzyme reaction site are typical for the interaction of duplexes both with DNA dependent ligases [45, 46] and polymerases [47].

Selectivity of enzyme conversion of bridged oligonucleotide complexes. The presence of the non-nucleotide insert in the substrate complexes formed with the bridged oligonucleotides can be considered as a disturbance of the DNA structure. There are literature data showing enhancement in the selectivity of some polymerases while using oligonucleotides containing modified units near the conversion site, which damage the regularity of the DNA helix [13, 48, 49]. Similar data for DNA ligases are absent. The presented results (Figs. 2 and 5) show that while using the native oligonucleotides, both DNA ligase and DNA polymerase are selective enough only when a mismatch is located near the 3′-end of the ligated oligonucleotide or the primer, respectively, i.e. in the proximity to the enzyme reaction site. For both enzymes the inhibition effect decreases as a mismatch is shifted from the 3′-end.

A completely different situation is observed for complexes based on bridged oligonucleotides (Figs. 2, 3, and 5). The presence of the non-nucleotide insert inside the enzyme-binding site of DNA-substrate or on its border leads to significant increase in the selectivity of the enzyme reaction. For example, the discrimination factors for the mismatch distant from the conversion site by six nucleotides (probe N6^6) amount to 8 and 12 in the ligation (Fig. 3a) and extension (Fig. 5c) reactions, respectively. In these cases the native probes do not allow reliable discrimination of a single-nucleotide substitution in the template strand; the discrimination factors are 2.9 and 1.2 for ligation and extension, respectively. The presence of the non-nucleotide insert is manifested even when a mismatch is distant from the modified site in the substrate complex. The efficiency of the discrimination of wrong base pairs nearer and nearer the 3′-end of the elongated oligomer drastically increases when the non-nucleotide loop is inserted in the polymerase-binding region. In this case, the insert located in the border region at a distance of six nucleotides (N6^6) provides the maximum selectivity of the enzyme action (the discrimination factor is 53; Fig. 5c).

It should be noted that such an influence of the distant insert is the result of the combination of two effects:

the relatively low inhibition in the perfect substrate complex and the pronounced inhibition in the mismatched complexes caused by the insert. For modified complexes containing the insert near the 3′-end of the primer, high selectivity of the extension is also observed although against the total decrease in their substrate properties (Fig. 5). The insert in the border of the ligase binding site, for example in the P component of the tandem, leads to enhancement, albeit less pronounced, in the efficiency of discrimination of single mismatches even when they are located in the duplex region of the OH component (Fig. 2).

It was mentioned above that replacement of the DEG insert in the modified OH component of the tandem N6^6 by any other studied insert (TF, PD, TEG, DD, or HEG) influences the efficiency of the formation of the full-size ligation product. It is important that the nature of the "border" insert can affect the efficiency of the mismatch discrimination (Fig. 3). The inserts such as DD and HEG noticeably deteriorate the substrate properties of complexes and, on the other hand, rather increase the sensitivity of DNA ligase to mismatches, while $(DEG)_5$ and TF reduce this characteristic.

The presence of the non-nucleotide loop in substrate duplexes formed from the bridged oligonucleotides is some kind of disturbance in the substrate structure, which in some way impedes the enzyme catalysis. The additional disturbance caused by a mismatch significantly reinforces this effect. Unlike native oligonucleotides, bridged oligomers more efficiently discriminate nucleotide discrepancies in a template in the presence of T4 DNA ligase and *Taq* DNA polymerase. The discrimination efficiency does not reduce even at the distance of a mismatch from the catalysis site.

Thus, the properties of the substrate complexes based on bridged oligonucleotides containing non-nucleotide inserts demonstrate that these oligomers are prospective tools for precise mapping of the sites of binding between DNA-dependent enzymes and substrates. They can also be used as highly selective probes for the determination of DNA sequences by means of molecular hybridization with participation of DNA-dependent enzymes.

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