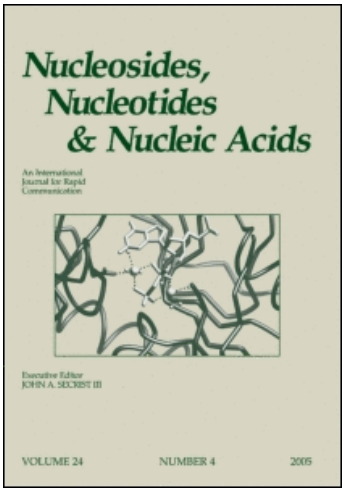

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A NEW STRATEGY OF DISCRIMINATION OF A POINT MUTATION BY TANDEM OF SHORT OLIGONUCLEOTIDES

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ABSRTACT: A new strategy based on the use of cooperative tandems of short oligonucleotide derivatives (TSOD) has been proposed to discriminate a "right" DNA target from a target containing a single nucleotide discrepancy. Modification of a DNA target by oligodeoxyribonucleotide reagents was used to characterize their interaction in the perfect and mismatched complexes. It is possible to detect any nucleotide changes in the binding sites of the target with the short oligonucleotide reagent. In the presence of flanking di-3',5'-N-(2-hydroxyethyl)phenazinium derivatives of short oligonucleotides (effectors) the tetranucleotide alkylating reagent modifies DNA target efficiently and site-specifically only in the perfect complex and practically does not modify it in the mismatched complex. It has been shown that TSOD is much more sensitive tool for the detection of a point mutation in DNA as compared to a longer oligonucleotides.

INTRODUCTION Biological processes with a participation of nucleic acids, detection of pathologic genes, study of DNA sequences, design of new drugs, etc., are based on the complementary interaction of oligonucleotides with nucleic acids. To provide the effective and site-specific interaction of an oligonucleotide and nucleic acid, extended oligonucleotides (more than 20-mers) are usually used. Such oligomers however form both perfect and imperfect (with a mismatched base pair or with a loop) complexes with the same stability under physiological conditions. The selective recognition of nucleic acids by extended oligonucleotides is a problem which hardly can be solved because it is impossible to change temperature and other conditions and regulate the process of the complex formation in the *in vivo* systems. Researchers try to develop a method for increasing selectivity of the oligonucleotides and nucleic acids interaction where the formation of imperfect complexes would be impossible [1-13].

For a few last years, we have been developing a new strategy of the efficient and site-specific modification of DNA targets by short oligonucleotide reagents in the presence

of auxiliary oligonucleotides, or effectors (short oligonucleotides or their 3',5'-di-N-(2-hydroxyethyl)-phenazinium derivatives) [14-18]. It was shown a reactive derivative of a short oligonucleotide such as tetramer can modify the target only when the whole tandem forms the complex with a target, while the reagent itself without effectors cannot do it [16, 19].

In this work the use of a tandem of short oligonucleotide derivatives (TSOD) is proposed to enhance the selectivity of recognition of the definite sites of nucleic acids and discriminate even a single nucleotide discrepancy.

MATERIALS AND METHODS

Synthesis of oligodeoxyribonucleotides and their derivatives

Oligodeoxyribonucleotides were synthesized using the phosphotriester method with 5'-*p*-chlorophenyl-*N*-acyl-3'-*O*-levulinyl-nucleoside 5'-phosphates as a nucleotide component and *p*-chlorophenyl-*β*-cyanoethyl-*N*-acyl-nucleoside 5'-phosphates as a nucleoside component [20].

3',5'-Di-N(2-hydroxyethyl)phenazinium derivatives of oligodeoxyribonucleotides were prepared as previously described [21].

Alkylating derivatives of oligodeoxyribonucleotides containing 4(N-2chloroethyl,N-methylamine)benzylmethylamide (RCl)p$\mathbf{N}_4$, (RCl)p$\mathbf{N}_8$, and (RCl)p$\mathbf{N}_{12}$ were synthesized according to the protocol [22] and were analyzed by HPLC on LiChrosorb RP-18 column.

Physical measurements

Concentration of oligonucleotides and their derivatives was calculated from the absorption value at 260 nm in UV spectra using ε_{260} of mono- and dinucleotides [23], alkylating group ($1.47 \cdot 10^4$ $M^{-1}cm^{-1}$) [24], and N-(2-hydroxyethyl)phenazinium residue ($1 \cdot 10^4$ $M^{-1}cm^{-1}$) [21]. The thermal denaturation of oligonucleotide complexes was carried out in buffer containing 0.01 M sodium cacodylate (pH 7.4), 0.1 M NaCl, and 1 mM EDTA using the UV detector of Milichrom liquid chromatograph in thermoregulated cell specially designed for this purpose. Concentration of each oligonucleotide component was 13 μM. The speed of heating was 0.5-0.7 °C/min. The equilibrium optical melting curves were constructed by 600-700 experimental points with the step of 10 points/°C and were utterly reversible in the processes of hitting-cooling. The melting curves were normalized to the value of the optical density corresponding to the highest temperature in the data set. The first derivatives of the melting curves by temperature are calculated using gradient of linear approximation by 10 experimental points. The melting curves of M1 · p$\mathbf{N}_4$ complexes in the presence of E1 and E2 effectors were calculated from a difference of melting curves of M1 · (E1+p$\mathbf{N}_4$+E2) complexes and of M1 · (E1+E2) complex. The T_{max} of the calculated curves were denoted as T_m of complexes M1 · p$\mathbf{N}_4$ in the presence of effectors [17, 19].

Modification of DNA targets with alkylating oligonucleotide derivatives

5'-^{32}P-Labeled oligonucleotides were prepared according to the protocol [25]. 3'-^{32}P-Labeled ssDNA-fragment (302-mer) was prepared by S.Mamaev (Novosibirsk Institute of Bioorganic Chemistry, Russia) [26].

Modification of M1 target (20-mer) by the alkylating reagents was carried out in buffer containing 0.01 M Tris-HCl (pH 7.2), 0.1 M NaCl, and 1 mM EDTA at 20 °C for 48 hours, at 25 °C for 25 hours, and at 37 °C for 8 hours (> 5 half-times of ionization of C-Cl-bond in the reagents) [24]. Concentration of M1 target in the reaction mixtures was 0.5 μM, concentration of each oligonucleotide derivative (the reagent or effector) was 10 μM.

Modification of 1 μM M2 or M3 targets was carried out in buffer containing 0.02 M Na_2HPO_4 (pH 7.4), 0.16 M NaCl, and 0.1 mM EDTA at 25 °C for 24 hours using 1 μM ssDNA targets and 50 μM of each oligonucleotide derivative (reagent or effector).

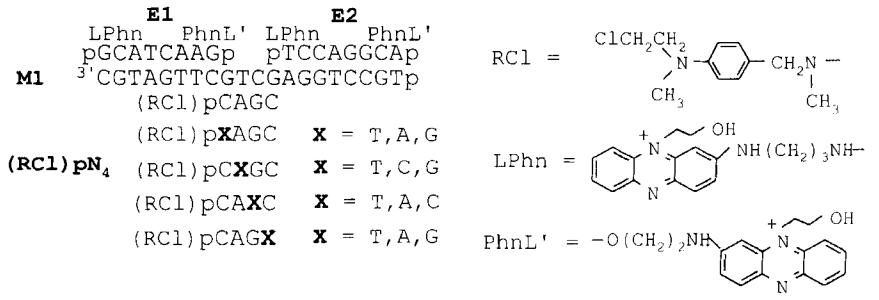
The modification of DNA fragment M4 (302-mer) was carried out in buffer containing 0.02 M Na_2HPO_4 (pH 7.4), 0.16 M NaCl, and 0.1 mM EDTA at 25 °C for 24 h using 50 μM of each oligonucleotide derivative (reagent or effector).

The reaction mixture was treated by 1 M piperidine at 100 °C for 30-50 min to cleave the modified DNA target at positions of the alkylated bases [27]. The cross-linked C^{13} base of M1 target was determined by the treatment of the modified target with hydrazine hydrate before the piperidine treatment (data not shown). The cleavage products of the M1-M3 or M4 targets were analyzed by gel electrophoresis in 20% or 8% polyacrylamide (7 M urea) respectively. The radioactive spots were cut out from the gel and counted by liquid scintillator on the counter Mark III (Nuclear Chicago, USA). The extent of modification was calculated as the ratio of radioactivity of the product spot to the sum of radioactivity of all spots in a lane.

RESULTS AND DISCUSSION

The ability of the tandem of short oligonucleotide derivatives to discriminate a point mutation is demonstrated with the results of modification of the DNA target in perfect and mismatched complexes by the alkylating derivatives of tetranucleotides in the presence of effectors. In order to estimate the selectivity of DNA target recognition by these reagents, the discrimination factor (FD) has been determined. It was calculated as the ratio of the extent of the modification of the target by the alkylating reagent in the perfect and mismatched complexes.

At first, we studied the alkylation of ^{32}P-labeled 20-mer oligodeoxyribonucleotide (M1) by 5'-[P-4(N-2-chloroethyl-N-methylamine)-benzylmethylamide] of tetranucleotide, (RCl)pN_4, in the presence of 3',5'-di-N-(2-hydroxyethyl)-phenazinium derivatives of the octanucleotides (E1 and E2 effectors):



The mismatched M1 · (RCl)p$\mathbf{N}_4$ complexes were designed by changing successively all of the nucleotides in tetranucleotide p$\mathbf{N}_4$. The efficiency of M1 target modification by the tetranucleotide reagent (RCl)pCAGC in the perfect M1 · (E1+(RCl)pCAGC+E2) complex and by wrong reagents (RCl)p**X**AGC, (RCl)pC**X**GC, (RCl)pCA**X**C, (RCl)pCAG**X** in the mismatched M1 · (E1+(RCl)p$\mathbf{N}_4$ +E2) complexes has been compared (FIG. 1 and TABLE).

The extent of the modification of the target by reagent (RCl)pCAGC in the perfect complex amounts to 55% at 25 °C. A drastic decrease of the level of modification is observed for all mismatched M1 · (RCl)p$\mathbf{N}_4$ complexes. The extent of modification was shown to depend on the position and the type of changing nucleotide. In the case of (RCl)p**X**AGC reagents no modification was observed when **X** was **G** (**G**·G mismatch); therefore the extent of modification could not exceed 0.1 % taking into account the accuracy of measurement of ^{32}P-radioactivity, so FD could be estimated as 500 in this case. This assumption is true in all cases when no product of modification is observed.

The wrong reagents (RCl)p**T**AGC and (RCl)p**A**AGC modified M1 target with a yield of 5 and 8% (FD = 11 and 7, respectively). The similar results were observed if the second nucleotide in the reagent was changed; no target modification was occurred in M1 · (RCl)pC**X**GC complex in the case of **G** · T mismatch (FD > 500). The introduction of any wrong nucleotide in the third position of the reagent resulted in no modification of the target in M1 · (RCl)pCA**X**C duplex; therefore, for all mismatches **G** · G, **T** · G, **A** · G, FD were more than 500. The mismatches in M1 · (RCl)pCAG**X** complexes were less discriminated. In such complexes a minimum decrease of the modification extent was detected in the complex with **A** · G mismatch (FD = 4), while **T** · G mismatch was the most discriminated mismatched pair (FD = 28). The above results demonstrate that discrimination of the same mismatch depends significantly on its position as it was shown for the **G** · G mismatch at the first and the forth positions (FD were 500 and 9, respectively). It should be noted that the least recognizable was mismatch the **A** · G independently on its position.

The decrease of the extent of the target modification in mismatched M1 · (RCl)p$\mathbf{N}_4$ complexes is probably a consequence of a low association of the target with the wrong reagent. To examine this assumption, the thermal stability of the M1 · p$\mathbf{N}_4$ complexes in the

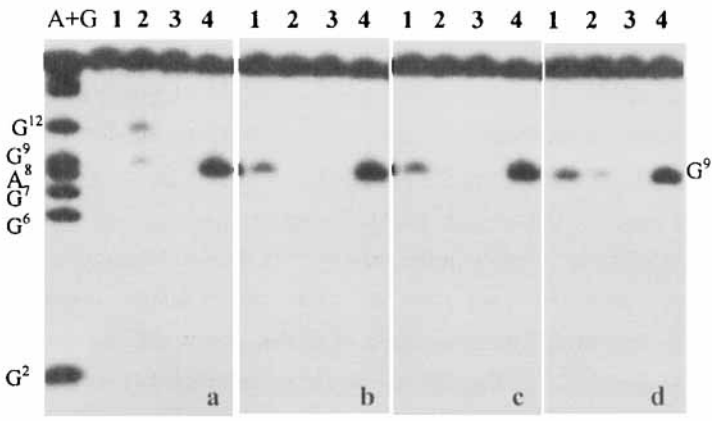


FIG. 1. Electrophoretic analysis of the modified M1 target (after piperidine treatment) in the matched (**4**) and mismatched (**1-3**) complexes with (RCl)p$\mathbf{N}_4$ in the presence of effectors E1+E2 at 25°C: a) (RCl)p**X**AGC, where **X** = T (**1**), A (**2**), G (**3**); b) (RCl)pC**X**GC, **X** = T (**1**), C (**2**), G (**3**); c) (RCl)pCA**X**C, **X** = T (**1**), A (**2**), C (**3**); d) (RCl)pCAG**X**, **X** = A (**1**), G (**2**), T (**3**); (RCl)pCAGC (**4**); (A+G) - M1 target products of the (A+G) sequencing reaction [28].

TABLE. The extent of M1 target modification in perfect and mismatched complexes by the alkylating derivatives of tetranucleotides (in the presence of E1 and E2 effectors), octanucleotides and dodecanucleotides, and their discrimination factors (FD)

Reagent	Mismatch	Extent of modification, %		FD		T_m ,°C*
		25°C	37°C	25°C	37°C	
(RCl)pCAGC**	-	55	42			38
(RCl)p**T**AGC	**T**·G	5	0	11	>400	8
(RCl)p**A**AGC	**A**·G	8	0	7	>400	< 0
(RCl)p**G**AGC	**G**·G	0	0	>500	>400	8
(RCl)pC**T**GC	**T**·T	14	0	4	>400	15
(RCl)pC**C**GC	**C**·T	5	0	11	>400	< 6
(RCl)pC**G**GC	**G**·T	0	0	>500	>400	22
(RCl)pCA**T**C	**T**·C	0	0	>500	>400	< 0
(RCl)pCA**A**C	**A**·C	0	0	>500	>400	< 0
(RCl)pCA**C**C	**C**·C	0	0	>500	>400	< 0
(RCl)pCAG**T**	**T**·G	2	0	28	>400	12
(RCl)pCAG**A**	**A**·G	14	0	4	>400	21
(RCl)pCAG**G**	**G**·G	6	0	9	>400	22
(RCl)p$\mathbf{N}_8$**	-	70	55			35
(RCl)p$\mathbf{N}_8$(A^4)	**A**·G	10	5	7	11	10
(RCl)p$\mathbf{N}_{12}$**	-	60	60			55
(RCl)p$\mathbf{N}_{12}$(A^4)	**A**·G	60	60	1	1	46

* Melting temperature of complexes formed by M1 target and the respective tetra- and oligonucleotides.

** The reagent which forms the perfect complex with M1 target.

presence of E1 and E2 effectors has been determined (FIG .2, TABLE). The melting curves of M1 · p**N**$_4$ complexes in the presence of E1+E2 effector pair were calculated by the difference in the melting curves of M1 · (E1+p**N**$_4$+E2) complexes and M1 · (E1+E2) complex [17, 19]. The melting temperature of the latter was equal to 55 °C [17]. While without the effectors any tetranucleotide p**N**$_4$ does not form the detectable complex with M1 target, in the presence of the effector pair, the perfect M1 · pCAGC complex has T_m = 38 °C. The cooperative melting of tetranucleotide complex in the presence of effectors is caused by the stacking interaction between adjusting base pairs of tetra- and octamers duplexes. Phenazinium residues of effectors intercalate in the nicks of double strand and additionally enhance the complex stability [19, 29]. The thermal stability of mismatched M1 · p**N**$_4$ complexes, however, is significantly lower (FIG. 2, TABLE).

The essential decrease of the melting temperature has been observed for M1 · p**X**AGC complexes with the mismatches at the first position of tetranucleotide. The complex with **A** · G mismatch is not formed at all; T_m of the complexes with **T** · G and **G** · G mismatches are below by ~30 °C, than T_m of the perfect M1 · pCAGC complex. The thermal stability of M1 · pC**X**GC complexes is decreased in the range of mismatches **C** · T > **T** · T > **G** · T. Any wrong nucleotide at the fourth position of the tetranucleotide causes the less significant destabilization of M1 · pCAG**X** complexes (T_m = 12-22 °C) in comparison with the other changes. The nucleotide replacement at the third position of the tetranucleotide is the most destructive and no M1·pCA**X**C complexes are detected. For all mismatched complexes a reduce of the thermal stability causes a decrease of a extent of target modification in comparison with the correct M1 · (RCl)pCAGC complex. The difference of T_{max} of complexes with the same mismatch (for example A·G in first or forth positions of tetramer complex) can be explained, probably, by the influence of nearest-neighbors [30]. Some observable discordance between the stability of imperfect tetranucleotide complexes and efficiency of modification of DNA target by reagents in corresponding complexes can be stipulated by features of a spatial organization of reactive complex [31, 32]. The influence of the type of a mismatch on the structure complexes formed by tetranucleotide reagent with target is confirmed for example by the following fact. In the mismatched M1 · p**X**AGC complexes the main point of the alkylation was the G^{12} base of M1 target, while in the other complexes the G^9 base was preferentially modified.

The analysis of the melting curves of M1 · p**N**$_4$ complexes (in the presence of E1 and E2) (FIG. 2) testifies that the formation of some mismatched *target·tetranucleotide* complexes is possible at 25 °C, while at higher temperatures (37 °C) the amount of imperfect M1 · p**N**$_4$ complexes is diminished to zero (FIG. 2a, b). At the same time, the extent of association of M1 target and the correct tetranucleotide, pCAGC, is reduced only to 0.5 (T_m of matched complex is 38 °C) (FIG. 2c). Therefore, the selectivity of the target modification by the tetranucleotide reagent in the presence of effectors can be enhanced at higher

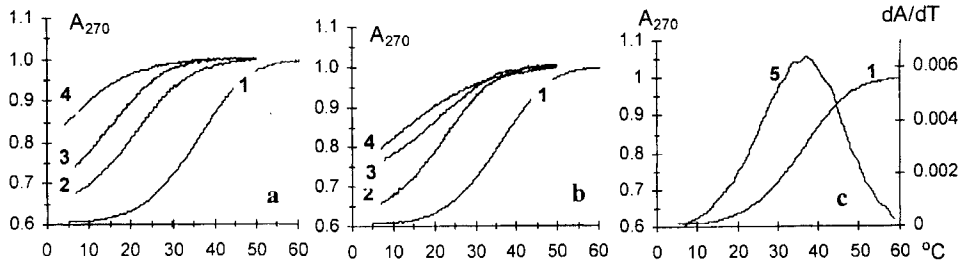


FIG. 2. The calculated melting curves of the matched (**1**) and mismatched (**2-4**) complexes M1 · p$\mathbf{N}_4$ in the presence of effectors E1+E2, where p$\mathbf{N}_4$ is: pCAGC (**1**); a) p**X**AGC, where **X** = T (**2**), G (**3**), A (**4**); b) pCAG**X**, X = G (**2**), A (**3**), T (**4**); c) differential melting curve of perfect complex M1 · pCAGC in the presence of effectors (**5**) (right axis).

temperatures. The extent of the modification of M1 target by reagent (RCl)pCAGC in the perfect complex is slightly reduced at higher temperature (42% at 37 °C vs. 55% at 25 °C), while no products of the modification are found for all mismatched M1·(RCl)p$\mathbf{N}_4$ complexes at 37 °C even with reagents (RCl)p**A**AGC, (RCl)pC**T**GC and (RCl)pCAG**A**, which are the least selective reagents at 25 °C (TABLE). The discrimination factors for all mismatched complexes at 37 °C exceed 400. Thus, TSOD can be used to modify the target at physiological temperature with high selectivity.

The ability of TSOD to discriminate mismatches in nucleic acid target was compared with the same ability of the extended oligonucleotides which have the 5'-terminal sequence pCAGC identical with the correct tetranucleotide in TSOD. The study was carried out using the same M1 target, octa- (p$\mathbf{N}_8$) and dodecanucleotide (p$\mathbf{N}_{12}$) and their alkylating derivatives ((RCl)p$\mathbf{N}_8$ and (RCl)p$\mathbf{N}_{12}$). The mismatched complexes have been prepared by changing the C to A base at the forth position of oligonucleotides. As it was described above, the similar replacement in tetranucleotide reagent (RCl)p$\mathbf{N}_4$ results in the least remarkable decrease of the modification of M1 target at 25 °C and completely prevents the modification at 37 °C.

The influence of the mismatch on the thermal stability of complexes formed by M1 target and p$\mathbf{N}_8$ and p$\mathbf{N}_{12}$ were examined (TABLE). The correct dodecanucleotide p$\mathbf{N}_{12}$ forms the most stable complex with M1 target (T_m is 56 °C). The **A** · G mismatch results in a decrease of the T_m value of M1 · p$\mathbf{N}_{12}$(A^4) complex by 9 °C. When the complexes are formed by octanucleotides p$\mathbf{N}_8$ and p$\mathbf{N}_8$(A^4), the difference in their T_m values is 25 °C. The obtained results allow us to assume, that selective modification of the DNA target may be possible only for the use of octanucleotide reagent.

The modification of M1 target by the alkylating derivatives of octa- and dodecanucleotides was carried out at 20 and 37 °C in the perfect complexes with reagents (RCl)p$\mathbf{N}_8$

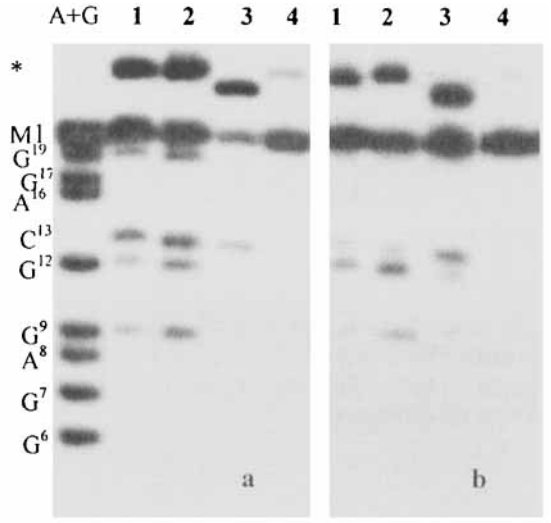


FIG. 3. Electrophoretic analysis of the modified M1 target (after piperidine treatment) at 25 °C (a) and 37 °C (b) from the matched (**1, 3**) and mismatched (**2, 4**) complexes with the reagents: (RCl)p$\mathbf{N}_{12}$ (**1**); (RCl)p$\mathbf{N}_{12}$(A^4) (**2**); (RCl)p$\mathbf{N}_8$ (**3**); (RCl)p$\mathbf{N}_8$(A^4) (**4**); (A+G) - M1 target products of the (A+G) sequencing reaction [28].
* - alkaline stable product of modification of C^{13} base of M1 target.

and (RCl)p$\mathbf{N}_{12}$ as well as in the mismatched complexes with (RCl)p$\mathbf{N}_8$(A^4) and (RCl)p$\mathbf{N}_{12}$(A^4) (TABLE and FIG. 3). At different temperatures the efficiency of M1 target modification by the dodecanucleotide reagents was shown to be identical both in the perfect and mismatched complexes (FD = 1). Obviously, it is stipulated by the sufficiently strong association of M1 target and wrong reagent (RCl)p$\mathbf{N}_{12}$(A^4) even at 37 °C. In contrast to dodecanucleotide, octanucleotide reagent can discriminate the perfect and mismatched complexes. At 25 °C the extent of alkylation amounts to 72% in perfect M1·(RCl)p$\mathbf{N}_8$ complex and only 10% in mismatched M1·(RCl)p$\mathbf{N}_8$(A^4) complex (FD = 7). The raise of the reaction temperature up to 37 °C results in increasing FD to 11. Showing a sufficient selectivity, the octanucleotide reagent, however, cannot modify the target site-specifically because octanucleotide has quite a few binding sites within a nucleic acid target. TSOD proposed in this work has been shown to posses the advantages both of short and prolonged oligonucleotide reagents (high selectivity and high site-specificity, respectively) because the reagent in the tandem system can modify DNA only if the complete *M1·effector-reagent-effector* complex is formed.

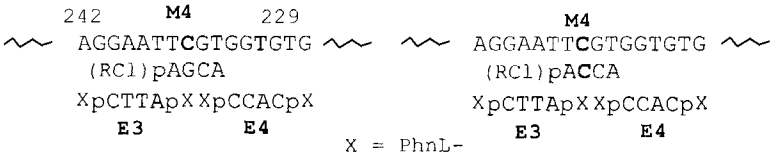
In order o confirm a possibility of the discrimination of a point mutation in DNA by TSOD, we compared the level of alkylation of the "correct" target DNA (M2) and "wrong" target (M3) in which one base in the binding site of the reagent was replaced:

```
   (RCl)pTTCA                          (RCl)pTTCA
3'TTTGCCAAGTCCCGTTp M2        3'TTTGCCATTGTCCCGTTp M3
  XpACGGpXXpGGGCpX                    XpACGGpXXpGGGCpX
```

X=LPhn

The extent of alkylation of the "correct" and "wrong" targets were 62 % and 9%, respectively, at 25 °C. These data confirm an opportunity of the selective modification of a target DNA using the *effector-reagent-effector* tandem even in the case of using diphenazinium derivatives of tetranucleotides as effectors. These data also testify a legitimacy of simulation of mismatched complexes by replacing monomers either in an address part of the reagent or in the target.

The possibility of the selective modification of large nucleic acids by TSOD has been also examined. For this purpose, modification of the DNA fragment (302-mer [27], M4 target) by the tetranucleotide reagent (RCl)pAGCA in the presence of two flanking E3 and E4 effectors (tetranucleotide diphenazinium derivatives) has been carried out. Tetranucleotide pAGCA has three binding sites within M4 target (in regions 36-39, 122-125 and 233-236) [16, 19]; the whole sequence of the *effector-reagent-effector* tandem is complementary to sequence 229-240 of M4 target:



It has been shown that the modification of M4 target by (RCl)pAGCA at 25 °C occurs site-specifically. The only G^{234} base, which is located in the binding site of the *effector-reagent-effector* tandem, is alkylated with a 53% yield. The mismatched tetranucleotide reagent (RCl)pACCA in which guanosine was replaced by cytidine at the second position has been also used. No product of modification is detected in this case (FIG.4). These data show that tetranucleotide reagent can modify DNA target only in the perfect complex and only in the region complementary to the complete tandem system *effector-reagent-effector.*

The presented results demonstrate that an extended oligonucleotide reagent providing unique binding with a definite site of DNA molecule cannot selectively modify DNA under physiological condition. The reactive derivatives of short oligonucleo-

FIG.4. Electrophoretic analysis of the modified M4 target (after piperidine treatment) at 25 °C in the presence of effectors E3 and E4 from the matched complex with reagent (RCl)pAGCA (**2**); from the mismatched complex with reagent (RCl)pACCA (**3**); native M4 target (**1**); (A+G) – M4 target products of the (A+G) sequencing reaction [28]. The structure of M4 target see in [26].

tides (e.g. octamer), in contrast to that, are not site-specific because of repeated binding sites with DNA; they are capable, however, of being selective reagents. The best way for the selective and site-specific modification of ssDNA is to use TSOD. Unlike extended oligonucleotide, TSOD can provide the more accurate recognition of DNA due to the higher selectivity of the interaction of short oligonucleotides. The site-specificity of recognition DNA by tetramer is provided by flanking effectors, because tetranucleotide can bind with DNA only where the complete complex *effector-tetramer-effector* with DNA is formed, so that the binding site of the complete tandem is equal to the binding site of an extended oligonucleotide of the summary length. The unique characteristics of the proposed approach can provide the high-sensitive recognition of a nucleic acid sequence and even discrimination of a point mutation. Both this fact and ability of TSOD to be the inhibit of HIV-1 proliferation in human lymphocyte culture [33] allows TSOD to be used for the design of potential therapeutic compounds.

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