# Sequence-specific RNA cleavage by oligonucleotide-peptide conjugates

*N. L. Mironova,[a,b] D. V. Pyshnyi,[a] E. M. Ivanova,[a] V. F. Zarytova,[a] M. A. Zenkova,[a]* H. J. Gross,[c] and V. V. Vlassov[a,b]*

[a]*Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences, 8 prosp. Akad. Lavrent´eva, 630090 Novosibirsk, Russian Federation. Fax: +7 (383 2) 33 3677. E-mail: marzen@niboch.nsc.ru*

[b]*Novosibirsk State University, 2 ul. Pirogova, 630090 Novosibirsk, Russian Federation.*

[c]*Institute of Biochemistry, Biocenter, D-97074 Wurzburg, Germany**

This paper considers the site-directed cleavage of the *in vitro* transcript of human $tRNA^{Lys}_3$ by the conjugate $^{C}NH_2Gly(ArgLeu)_3ArgLeu^{N}$—p-DEG—$^{5'}$CCCTGGACCCTCAGAT$^{3'}$ (conjugate *pep*-1A) of the oligodeoxyribonucleotide 1A (CCCTGGACCCTCAGAT) with a peptide $[LeuArg]_4Gly$ attached at the 5´ terminus of the oligonucleotide through diethylene glycol as a linker. Under physiological conditions, the conjugate *pep*-1A cleaved the C56-A57 phosphodiester bond of the RNA target with high efficacy. In addition, the conjugate cleaved several other bonds in the D loop, at the basis of the amino-acceptor stem, and in the anticodon hairpin due, probably, to the formation of imperfect complexes of the conjugate with partially complementary sequences in the RNA structure.

**Key words:** oligonucleotide-peptide conjugate, synthetic ribonucleases, $tRNA^{Lys}_3$.

The development of approaches to the sequence-specific RNA cleavage is a topical problem arising from both the necessity of manipulating with RNA molecules in molecular biological studies and the demand for the design of therapeutic agents inactivating RNAs of infectious agents and mRNAs which encode proteins responsible for the development of pathological states. An approach to the design of compounds capable of cleaving particular RNAs involves the construction of oligonucleotide conjugates with catalytically active groups, which can induce cleavage of the phosphodiester bonds in RNA. The oligonucleotides ensure highly selective interactions of conjugates with RNA and deliver catalytically active groups to the desired region of the target molecule.

There are several types of oligodeoxyribonucleotide conjugates bearing groups, which are capable of catalyzing RNA cleavage. Among these are imidazole residues,[1,2] imidazole derivatives bearing an amino group,[3] bisimidazole constructs,[4] ethylenediamine, propylenediamine,[5] terpyridine derivatives chelating copper ions,[6] and various lanthanide complexes.[7] The above-mentioned oligonucleotide conjugates both with metal complexes as active groups and with organic RNA-hydrolyzing constructions cleave RNA with different efficacies. Thus the degree of RNA cleavage was ~2—5%, 55—80%, and 80—90% upon its incubation for *ca*. 1 day using conjugates with imidazole and $Zn^{2+}$ ions as a cofactor,[1,8] the texaphyrin complex with the $Dy^{3+}$ cofactor,[9,10] and macrocyclic europium complexes, respectively.[11,12] Conjugates containing bisimidazole catalytic constructs, which mimic the active centers of natural ribonucleases, proved to be more efficient.[4,13—16]

Screening of peptides with different structures revealed simple structures capable of cleaving RNA,[17] *viz*., the $[LeuArg]_n$ peptides with alternating hydrophobic and basic amino acid residues.[18,19] The attachment of short peptides $[LeuArg]_{2—4}$ to the 5´-terminal phosphate of the 6-mer oligonucleotide resulted in conjugates capable of cleaving RNA.[20]

In the present study, we examined the site-directed cleavage of the *in vitro* transcript of human $tRNA^{Lys}_3$ by the oligonucleotide-peptide conjugate $^{C}NH_2Gly(ArgLeu)_3ArgLeu^{N}$—p-DEG—$^{5'}$CCCTGGACCCTCAGAT$^{3'}$ (*pep*-1A). It was demonstrated that this conjugate binds to several regions of RNA under physiological conditions. The cleavage of the *in vitro* transcript of human $tRNA^{Lys}_3$ in the presence of *pep*-1A occurs at the C56—A57 phosphodiester bond in the vicinity of the oligonucleotide binding site and also at several other phosphodiester bonds in the region of the D loop and the anticodon hairpin in the vicinity of the sequences partially complementary to the oligonucleotide.

## Experimental

Tris(hydroxymethyl)aminomethane (Tris), acrylamide, *N*,*N*´-methylenebisacrylamide, agarose, ATP, DTT (dithiothreitol), BSA (bovine serum albumin), and HEPES were pur-

* Institut für Biochemie, Biozentrum, Am Hubland, D-97074 Wurzburg, Deutchland.

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 7, pp. 1088—1096, July, 2002.

1066-5285/02/5107-1177 $27.00 

chased from Sigma (USA). Other reagents of "special purity" grade or "reagent" grade were domestically produced.

[γ-$^{32}$P]Adenosine 5′-triphosphate ([γ-$^{32}$P]ATP) (3000 Ci mmol$^{-1}$) and [5′-$^{32}$P]cytidine 3′,5′-bisphosphate ([$^{32}$P]pCp) (3000 Ci mmol$^{-1}$) were from Biosan (Russia), T4 RNA ligase was purchased from Fermentas (Lithuania), the oligopeptide [LeuArg]$_4$Gly-amide was produced at the "Vector" State Scientific Center (Russia), RNase T1 was from Boehringer Mannheim (Germany), and restriction endonuclease $Zsp_2I$ was purchased from Sibenzim (Russia); T7 RNA polymerase was produced at the Novosibirsk Institute of Bioorganic Chemistry of the Siberian Branch of the Russian Academy of Sciences.

All biochemical assays were carried out in Milli-Q purified water (Millipore, USA). All buffer solutions were filtered through nitrocellulose filters (0.22 μm pore size; Millipore, USA). [$^{32}$P]-Labeled RNA was visualized in gel by autoradiography on a Renex X-ray film (Russia).

The oligodeoxyribonucleotide 1A with the spacer, *viz.*, p(DEG)CCCTGGACCCTCAGAT, was synthesized by the phosphoramidite method on an automated ASM-700 synthesizer (Biosset, Russia). After deprotection, the oligonucleotide was isolated by successive ion-exchange and reversed-phase HPLC on columns packed with Polysil SA-500 (Teoreticheskaya praktika [Theoretical Practice], Russia) and LiChrosorb RP-18 (Merck).

The conjugate *pep*-1A was synthesized according to a procedure reported previously.[21] After the synthesis, the conjugate was isolated by reversed-phase HPLC on a column with LiChrosorb RP-18. The homogeneity of the oligonucleotide 1A and conjugate *pep*-1A were analyzed by electrophoresis in 20% denaturing polyacrylamide gel (PAAG) after which the nucleotide material was stained with the Stains-all dye. The homogeneity of the specimens used in subsequent studies was no lower than 98%.

**Linearization of plasmid pHtk(wt) with restriction endonuclease $Zsp_2I$.** The reaction mixture (400 μL) contained 10 *mM* Tris-HCl, pH 7.6, 10 *mM* $MgCl_2$, 1 *mM* DTT, and plasmid pHtk(wt) (100 μg). Restriction endonuclease $Zsp_2I$ was added to the reaction mixture (1.5 IU per μg of plasmid DNA) and the mixture was incubated at 37 °C for 4 h. The extent of hydrolysis of pHtk(wt) DNA was checked by electrophoresis in 1% agarose gel. After the reaction was quenched, the plasmid DNA was extracted with an equal volume of a 1 : 1 phenol—chloroform mixture, precipitated with ethanol from 0.3 *M* sodium acetate (pH 5.8), kept at −20 °C for 16 h, and centrifuged (15 min, 4 °C, 13000 rpm). The precipitate was washed with 80% ethanol, dried, and dissolved in water (the concentration was 1 μg μL$^{-1}$).

**Preparation of the *in vitro* transcript of $tRNA^{Lys}_3$.** The *in vitro* transcript of $tRNA^{Lys}_3$ was prepared according to a standard procedure using T7 RNA polymerase.[22] The reaction mixture (300 μL) contained 40 *mM* Tris-HCl, pH 7.5, 6 *mM* $MgCl_2$, 2 *mM* spermidine, 10 *mM* NaCl, 10 *mM* DTT, 3 *mM* ribonucleoside triphosphates each (ATP, UTP, CTP, GTP), linearized plasmid pHtk(wt)/$Zsp_2I$ (30 μg), and T7 RNA polymerase (3 IU per μg of linearized DNA). The reaction mixture was incubated at 37 °C for 2 h. After the reaction was quenched, the transcript was twice extracted with an equal volume of a 1 : 1 phenol—chloroform mixture. After the addition of 3 *M* sodium acetate (0.1 volume; pH 5.8) to the aqueous phase, RNA was precipitated with ethanol and kept at −20 °C for 16 h. The RNA pellet was recovered by centrifugation (10 min, 13000 rpm), washed with 80% ethanol, dissolved in 8 *M* urea containing 0.05% Bromophenol Blue and 0.05% Xylene Cyanol, and applied onto 8% denaturing PAAG. RNA in the gel was visualized by UV-shadow using an intensifying screen (Merck). RNA was eluted from the gel with 0.3 *M* sodium acetate, pH 5.8, in the presence of 10% phenol (300 μL) and then precipitated with a fourfold volume of ethanol. The RNA pellet was centrifuged, washed with 80% ethanol, dried, and dissolved in water. The concentration of the RNA transcript was determined from the optical density at 260 nm. The RNA transcript was stored in water at −20 °C.

**Preparation of the [3′-$^{32}$P]-labeled *in vitro* transcript of $tRNA^{Lys}_3$.** The tRNA was labeled using [$^{32}$P]pCp and T4 RNA ligase. The reaction mixture (10 μL) containing 0.1 $OU_{260}$ of the *in vitro* transcript of $tRNA^{Lys}$, 10 IU T4 RNA ligase, 20 MBq [$^{32}$P]pCp, 50 *mM* HEPES-KOH, pH 7.5, 10 *mM* $MgCl_2$, 2 *mM* DTT, BSA (0.1 mg mL$^{-1}$), 0.1 *mM* ATP, and 10% DMSO was incubated at 4 °C for 18 h. The labeled RNA was isolated by electrophoresis in 8% PAAG containing 8 *M* urea. Then RNA was eluted with 0.5 *M* ammonium acetate containing 0.1% SDS and 1 *mM* EDTA (300 μL), precipitated with ethanol, dissolved in water, and stored at −20 °C. The specific activity of the labeled $tRNA^{Lys}_3$ thus obtained was 150000—200000 cpm pmol$^{-1}$.

**Cleavage of RNA by the oligonucleotide-peptide conjugate.** The reaction mixture (10 μL) containing [3′-$^{32}$P]$tRNA^{Lys}_3$ (50000 cpm) and the conjugate *pep*-1A (5—50 μmol L$^{-1}$) was incubated in 50 *mM* Tris-HCl, pH 7.0, containing 0.2 *M* KCl, 1 *mM* EDTA, and *E. coli* total tRNA (0.1 mg mL$^{-1}$) at 37 °C for 42 h. The reaction was quenched by precipitation with 2% lithium perchlorate solution in acetone (100 μL). The RNA pellet was separated by centrifugation, washed with acetone (100 μL), and dissolved in a loading buffer which contained 6 *M* urea and leading dyes. The hydrolysis products were analyzed by electrophoresis in 12% PAAG containing 8 *M* urea. The determination of the hydrolysis sites was made by comparing with the products of random hydrolysis of the [3′-$^{32}$P]-labeled $tRNA^{Lys}_3$ by RNase T1 and 2 *M* imidazole.[23]

**Study of the temperature dependence on the hydrolytic activity of the oligonucleotide-peptide conjugate.** The reaction mixtures (100 μL) containing [3′-$^{32}$P]$tRNA^{Lys}_3$ (500000 cpm), the conjugate *pep*-1A (10 μmol L$^{-1}$), 50 *mM* Tris-HCl, pH 7.0, 0.2 *M* KCl, 1 *mM* EDTA, and *E. coli* total RNA as a carrier (0.1 mg mL$^{-1}$) was incubated at 37, 40, 42, 46, and 50 °C over different periods of time. The hydrolysis products were analyzed as described above.

**Study of hybridization of the transcript of $tRNA^{Lys}_3$ with the conjugate *pep*-1A and oligonucleotide 1A.** Hybridization of the transcript of $tRNA^{Lys}_3$ with the conjugate *pep*-1A and oligonucleotide 1A was studied by the gel retardation method. The reaction mixture (10 μL) contained 0.5 μ*M* [3′-$^{32}$P]$tRNA^{Lys}_3$, 50 *mM* Tris-HCl, pH 7.0, 0.2 *M* KCl, 1 *mM* EDTA, *E. coli* total RNA (0.1 mg mL$^{-1}$), (±) 5 *mM* $MgCl_2$, and 0.05—10 μ*M* oligonucleotide 1A or conjugate *pep*-1A. The reaction was carried out at 37 °C for 30 min. Then the samples were mixed with 8 μL of the loading buffer (20% Ficoll, 0.025% Bromophenol Blue, and 0.025% Xylene Cyanol) and immediately applied onto a running 10% native PAAG. Electrophoresis was carried out in a 100 *mM* Tris-borate buffer (pH 8.3) at 4 °C and

20 V $cm^{-1}$ for 6 h. After electrophoresis, the gel was dried and autoradiographed. The bands of free RNA and its complexes with the oligonucleotide and conjugate were cut out from the gel and the Cherenkov radioactivity was measured.

**Probing of the $tRNA^{Lys}_3$ structure in a complex with the oligonucleotide 1A using RNase T1.** The complex was hydrolyzed with RNase T1 under native and partially denaturing conditions. To perform hydrolysis under native conditions, the reaction mixture (10 μL) containing [3′-$^{32}$P]$tRNA^{Lys}_3$ (50000 cpm), 50 *mM* Tris-HCl, pH 7.0, 0.2 *M* KCl, 1 *mM* EDTA, *E. coli* total RNA (0.1 mg $mL^{-1}$), and the oligonucleotide 1A (1—10 μmol $L^{-1}$) was incubated at 37 °C for 30 min. To perform hydrolysis under partially denaturing conditions, 0.2 *M* KCl was excluded from the reaction mixture. Then 0.1—0.4 IU RNase T1 (0.2 IU $\mu L^{-1}$) was added to the sample thus prepared and the mixtures were incubated at 37 °C for 15 min. The reaction was quenched by precipitation of RNA with ethanol in the presence of 0.3 *M* sodium acetate. The hydrolysis products were analyzed in 12% PAAG containing 8 *M* urea. The determination of the hydrolysis sites was made by comparing with the products of random hydrolysis of the [3′-$^{32}$P]-labeled transcript of $tRNA^{Lys}_3$ by RNase T1 and 2 *M* imidazole.[23]

**Probing of the $tRNA^{Lys}_3$ structure in the $tRNA^{Lys}_3$-oligonucleotide 1A complex using RNase H.** Three reaction mixtures (10 μL each) containing [3′-$^{32}$P]$tRNA^{Lys}_3$ (50000 cpm), 50 *mM* Tris-HCl, pH 7.5, 0.2 *M* KCl, 1 *mM* EDTA, 1 *mM* DDT, and the oligonucleotide 1A (1—10 μmol L) were incubated at 37 °C for 30 min. Then 1 IU RNase H (1 IU $\mu L^{-1}$) was added to one sample and the subsequent dilutions of the enzyme were obtained by adding aliquots (1 μL) from the first reaction mixture to the second mixture and from the second mixture to the third mixture. Solutions of RNase H with concentrations of 100, 10, and 1 IU $mL^{-1}$ were thus obtained. The samples were incubated at 37 °C for 15 min. The reaction was terminated by precipitation of RNA with ethanol in the presence of 0.3 *M* sodium acetate. The hydrolysis products were analyzed in 12% PAAG containing 8 *M* urea as described above.

## Results and Discussion

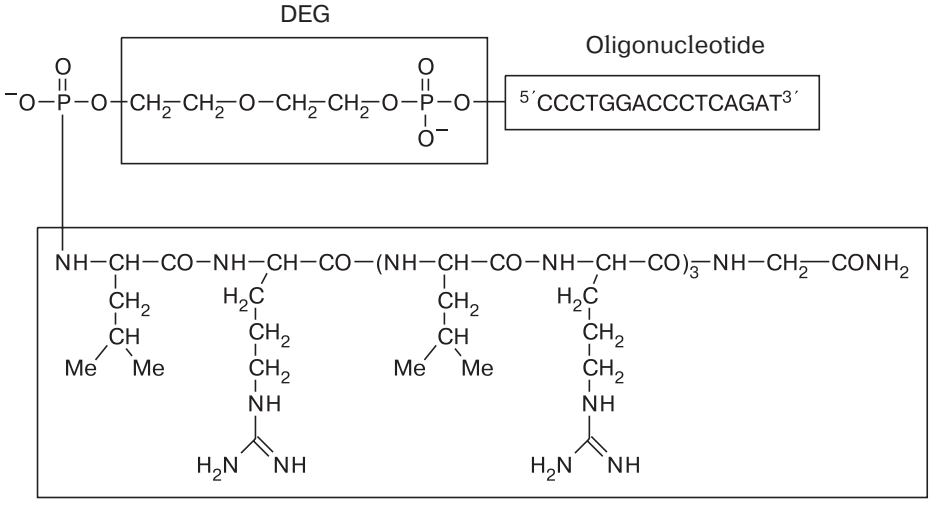
### Synthesis of oligonucleotide-peptide conjugates

Hexadecadeoxyribonucleotide p(DEG)—CCCTGGACCCTCAGAT (1A) bearing the diethylene glycol (DEG) residue attached to the 5′-terminus of the oligonucleotide through a phosphate group was synthesized according to a standard procedure.[21] The conjugate $^{C}$Gly(ArgLeu)$_3$ArgLeu$^{N}$—p-DEG—$^{5'}$CCCTGGACCCTCAGAT$^{3'}$ was prepared according to a procedure reported previously.[22] The peptide was attached to the oligonucleotide according to a procedure[22] involving the formation of the phosphoramide bond. The structure of the conjugate is shown in Scheme 1.

### Study of interaction of the conjugate *pep*-1A with the *in vitro* transcript of human $tRNA^{Lys}_3$

We used the *in vitro* transcript of human $tRNA^{Lys}_3$ as a model RNA (Fig. 1). The natural analog of this transcript serves as a primer for HIV-1 reverse transcriptase in the key step of virus cycle of replication.[24] The hybridization of the 3′-terminus of $tRNA^{Lys}_3$ with the primer binding site (PBS) of HIV-1 genomic RNA results in a pseudoknot whose tertiary structure is recognized by virus reverse transcriptase. Destruction of the tertiary structure of this pseudoknot blocks the reverse transcription of viral genomic RNA.[25] The conjugate was synthesized with the use of the oligonucleotide 1A complementary to the sequence 38—53 of $tRNA^{Lys}_3$. This oligonucleotide should deliver the peptide $[LeuArg]_4Gly$ to the C56—A57 bond located in the TΨC loop of the molecule (see Fig. 1).

**Scheme 1**



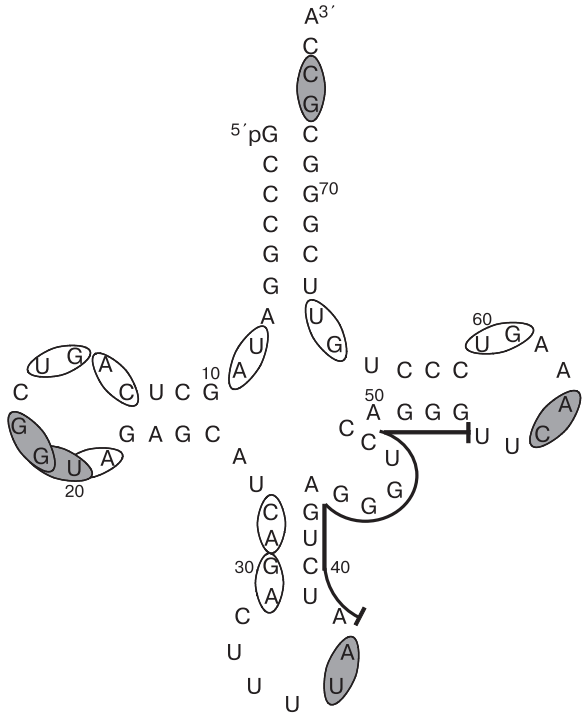


**Fig. 1.** Secondary structure of the *in vitro* transcript of human $tRNA^{Lys}_3$.[24] The phosphodiester bonds cleaved by the oligonucleotide-peptide conjugate *pep*-1A are marked by ovals. The intensity of shading corresponds to the extent of cleavage of the corresponding phosphodiester bond.

We studied the binding of $tRNA^{Lys}_3$ to the conjugate *pep*-1A and oligonucleotide 1A by the gel retardation method (Fig. 2). For this purpose, $tRNA^{Lys}_3$ was incubated in a standard buffer (see the Experimental section) in the presence or in the absence of 5 *mM* $MgCl_2$. Binding of the tRNA both to the oligonucleotide 1A (Fig. 2, *a*) and to the conjugate *pep*-1A (Fig. 2, *b*) resulted in several types of complexes characterized by different electrophoretic mobilities in 10% native PAAG. In complex **1**, $tRNA^{Lys}_3$ was bound to one molecule of the oligonucleotide 1A or conjugate *pep*-1A, whereas $tRNA^{Lys}_3$ in complex **2** binds at least two molecules of the oligonucleotide or conjugate. Previously, we have observed cooperative binding of two oligonucleotide molecules to RNA with the simultaneous formation of perfect and imperfect heteroduplexes in the case of yeast $tRNA^{Phe}$ (see Ref. 26) and the 23S rRNA fragment containing the α-sarcin loop.[27]

In the absence of magnesium ions, the extent of hybridization reached 100% when $tRNA^{Lys}_3$ and the oligonucleotide 1A were taken in equimolar concentrations (0.5 μmol $L^{-1}$). However, several types of complexes simultaneously formed under these conditions. In the presence of magnesium ions, complete binding of the oligonucleotide 1A was observed at the oligonucleotide concentration of 5 μmol $L^{-1}$. In the latter case, the formation of several types of complexes was also observed. Binding of the conjugate *pep*-1A to $tRNA^{Lys}_3$ occurred less efficiently but (as in the case of the oligonucleotide) several types of complexes were formed as well. The perfect duplex was predominantly formed only when the conjugate was taken at a concentration ranging from 0.1 to 0.7 μmol $L^{-1}$. The conjugate *pep*-1A was completely bound to $tRNA^{Lys}_3$ at the conjugate concentration of 1 μmol $L^{-1}$. In the presence of magnesium ions, binding of the conjugate to $tRNA^{Lys}_3$ is less efficient but several types of complexes were formed in the latter case as well. As the concentration of the oligonucleotide 1A or conjugate *pep*-1A was increased, the proportion of complex **2** gradually increased. Apparently, binding of the oligonucleotide 1A and conjugate *pep*-1A, even at low concentrations, to the 38—53 region of $tRNA^{Lys}_3$ causes a change in the structure of the $tRNA^{Lys}_3$ molecule providing efficient cooperative binding of one more oligonucleotide or conjugate molecule.

The results of the $tRNA^{Lys}_3$—oligonucleotide 1A probing with RNase T1 are presented in Fig. 3. It can be seen that in the $tRNA^{Lys}_3$–oligonucleotide 1A complex, the phosphodiester bonds adjacent to the G53, G52, G51,

**Table 1.** Context analysis of binding sites of the oligonucleotide 1A to $tRNA^{Lys}_3$

| Region of $tRNA^{Lys}_3$ | Imperfect complex* |
|---|---|
| **17—2** | $^{1}$CC**CTGG**AC**C**CC**TC**A**G**AT$^{16}$<br>$^{17}$CU**GACU**C**G**AU**AG**G**C**CC$_{2}$ |
| **26—11** | $^{1}$CC**CT**GG**ACC**CT**C**A**GA**T$^{16}$<br>$^{26}$AC**GA**GA**UGG**CU**G**A**CU**C$_{11}$ |
| **32—17** | $^{1}$CC**CTGG**AC**C**C**TC**AGAT$^{16}$<br>$^{32}$CA**GACU**AC**G**A**GA**UGGC$_{17}$ |
| **33—18** | $^{1}$CCC**T**G**GA**CC**CTC**A**GA**T$^{16}$<br>$^{33}$UCA**G**A**CU**AC**GAG**A**U**G**G$_{18}$ |
| **41—26** | $^{1}$CCC**T**G**GA**CCC**TC**A**GAT**$^{16}$<br>$^{41}$UCU**AA**U**U**TTC**AG**A**CUA**$_{26}$ |
| **66—51** | $^{1}$C**C**CT**GGAC**CCTC**A**GA**T**$^{16}$<br>$^{66}$U**G**UC**CUG**AACU**U**GG**G**$_{51}$ |

* The program ignored the possibility of the formation of bulge-loops in the oligonucleotide upon the formation of imperfect complexes; 1—16, the sequence of the oligonucleotide 1A from the 5′-terminus to the 3′-terminus; the sequence of the $tRNA^{Lys}_3$ region in a reverse orientation is shown in the second row; the possible base pairs are connected by lines. The nucleotides forming complementary pairs are given in bold type.

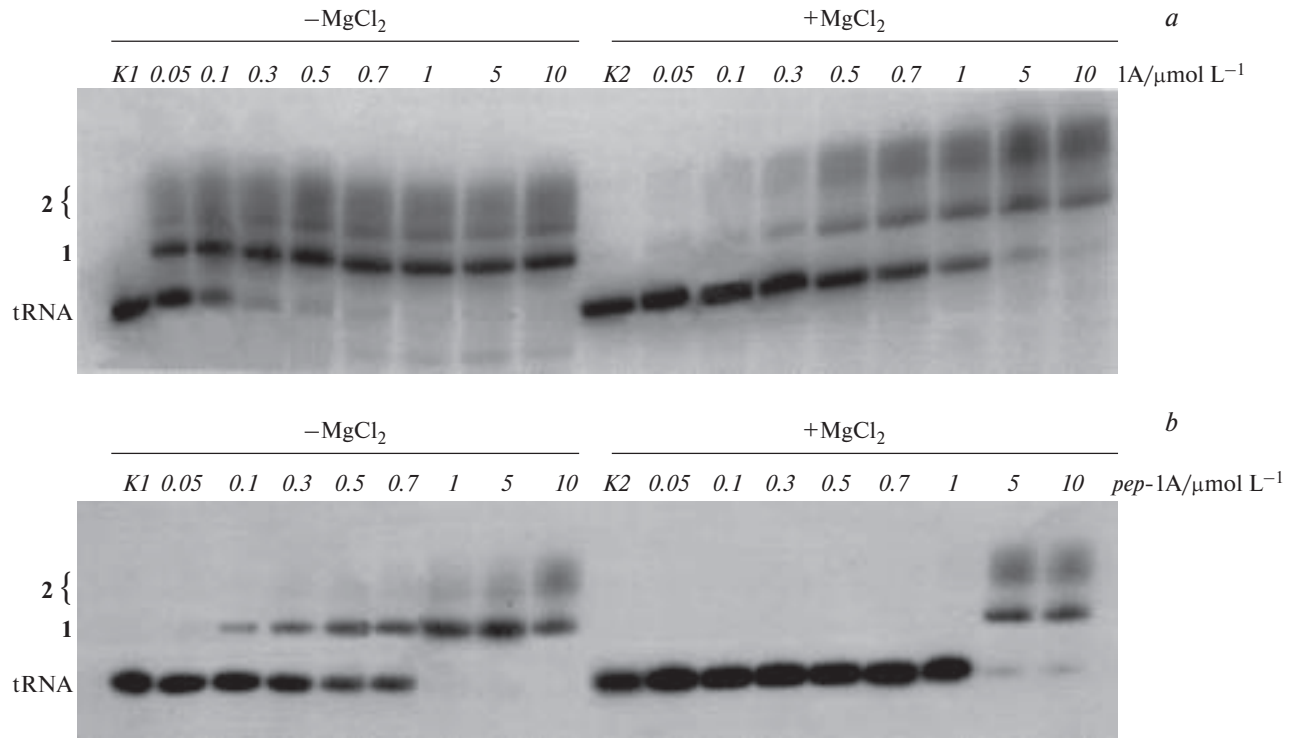



**Fig. 2.** Analysis of $tRNA^{Lys}_3$ binding to the oligonucleotide 1A (*a*) and the conjugate *pep*-1A (*b*) by the gel retardation method. The autoradiograph of 10% native PAAG. *a*. Incubation of $tRNA^{Lys}_3$ under standard conditions in the presence of the oligonucleotide 1A (0.05—10 μmol $L^{-1}$) at 37 °C for 30 min. Lanes *K1* and *K2*, $tRNA^{Lys}_3$ in the absence of the oligonucleotide 1A; lanes *0.05—10* ($-MgCl_2$), incubation of $tRNA^{Lys}_3$ with the oligonucleotide 1A in the absence of magnesium ions. Lanes *0.05—10* ($+MgCl_2$), incubation of RNA with the oligonucleotide 1A in the presence of magnesium ions (5 μmol $L^{-1}$). *b*. Incubation of $tRNA^{Lys}_3$ under the standard conditions in the presence of 0.05—10 *μM* conjugate at 37 °C for 30 min. Lanes *K1* and *K2*, $tRNA^{Lys}_3$ in the absence of the conjugate *pep*-1A; lanes *0.05—10* ($-MgCl_2$), incubation of $tRNA^{Lys}_3$ with the conjugate *pep*-1A in the absence of magnesium ions. Lanes *0.05—10* ($+MgCl_2$), incubation of $tRNA^{Lys}_3$ with the conjugate *pep*-1A in the presence of magnesium ions (5 μmol $L^{-1}$).

G46, G45, G44, and G42 bases, which match completely the oligonucleotide, are protected from hydrolysis with RNase T1 under the native conditions. Simultaneously, the extent of hydrolysis at the G65, G30, and G18, G19, G22, G24 residues in the D loop is sharply increased. In addition, sensitivity of G10 to hydrolysis is slightly changed. These results provide evidence that binding of the oligonucleotide 1A to the 38—53 region is accompanied by a substantial reorganization of the $tRNA^{Lys}_3$ molecule as a whole (particularly, in the region of the D hairpin). The probing of the complex with the use of RNase H revealed the formation of the perfect heteroduplex with the oligonucleotide 1A only in the 38—53 region of $tRNA^{Lys}_3$ (the data are not presented). It is known that RNase H recognizes heteroduplexes consisting of at least six base pairs.[28] Apparently, imperfect complexes contain shorter continuous heteroduplexes.

The context analysis of the $tRNA^{Lys}_3$ sequence showed that the oligonucleotide 1A can form at least two imperfect complexes in the region of the D loop, one imperfect complex in the region of the anticodon loop, and one imperfect complex in the region of the TΨC loop in addition to the 16-mer complementarity region 38—53 (Table 1).

### Ribonuclease activity of the oligonucleotide-peptide conjugates

The ribonuclease activity of the conjugate *pep*-1A was assayed under physiological conditions (37 °C, pH 7.0). The products of [3′-$^{32}$P]-$tRNA^{Lys}_3$ hydrolysis by the conjugate *pep*-1A were analyzed by electrophoresis in 12% PAAG under denaturing conditions (Fig. 4). Incubation of RNA in the presence of the oligonucleotide 1A (Fig. 4, lane *6*) or the peptide $[LeuArg]_4Gly$ taken in a concentration of 10 μmol $L^{-1}$ (Fig. 4, lane *5*) (incubation for 24 h) did not lead to RNA cleavage. When the concentration of the peptide was increased to 50 μmol $L^{-1}$, aggregation with RNA occured to give conjugates stable under denaturing conditions (results are not presented). In the presence of 10 μ*M* conjugate

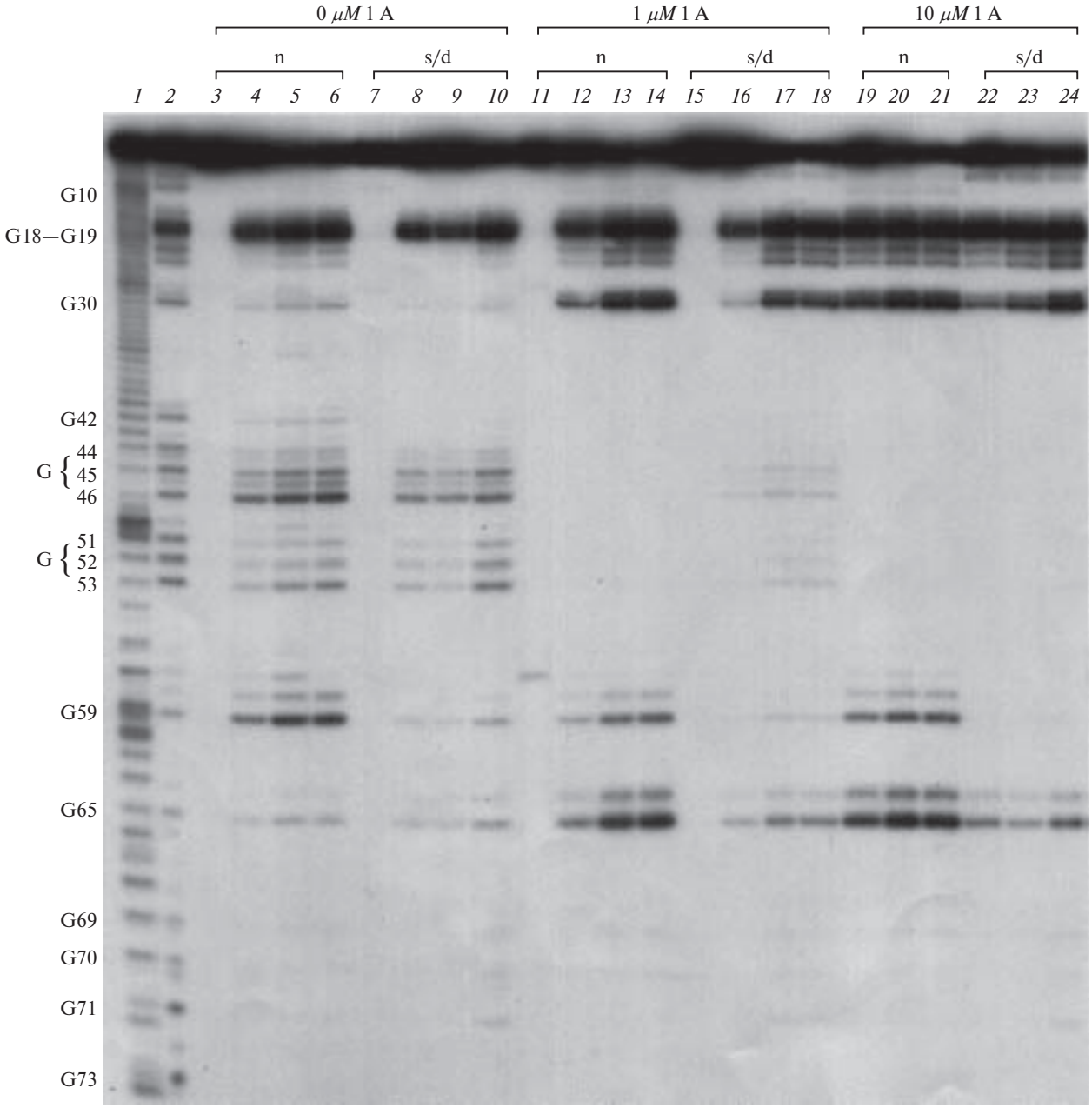



**Fig. 3**. Probing of the complex of 3′-[$^{32}$P]—tRNA$^{Lys}_3$ with the oligonucleotide 1A using RNase T1. The autoradiograph of 12% PAAG containing 8 *M* urea. The concentration of the oligonucleotide 1A for each lane is given at the top of the figure: *1* and *2*, hydrolysis of tRNA$^{Lys}_3$ with 2 *M* imidazole and RNase T1 under the denaturing conditions, respectively; *3*, *7*, *11*, and *15*, incubation of tRNA$^{Lys}_3$ in the absence of RNase T1; *4*, *8*, *12*, *16*, *19*, and *22*, incubation of tRNA$^{Lys}_3$ in the presence of 0.1 IU RNase T1; *5*, *9*, *13*, *17*, *20*, and *23*, incubation of tRNA$^{Lys}_3$ in the presence of 0.2 IU. RNase T1; *6*, *10*, *14*, *18*, *21*, and *24*, incubation of tRNA in the presence of 0.4 IU RNase T1 for 10 min; n, native conditions, s/d, incubation in the absence of monovalent and divalent cations.

*pep*-1A, the site-directed RNA cleavage occurs under the conditions of complete binding of RNA (after 3 h) at the C56—A57 bond located in the vicinity of the probable localization of the peptide residue upon the formation of the full-sized complementary heteroduplex. An increase in the incubation time (lanes *9*, *10*) or the concentration of the conjugate (lanes *11—14*) led not only to an increase in the yield of the hydrolysis products in the C56—A57 region but also to the formation of a limited series of products of RNA hydrolysis at the G73—C74 phosphodiester bond in the amino-acceptor stem, at the G65—U66 and G59—U60 bonds in the TΨC hair-

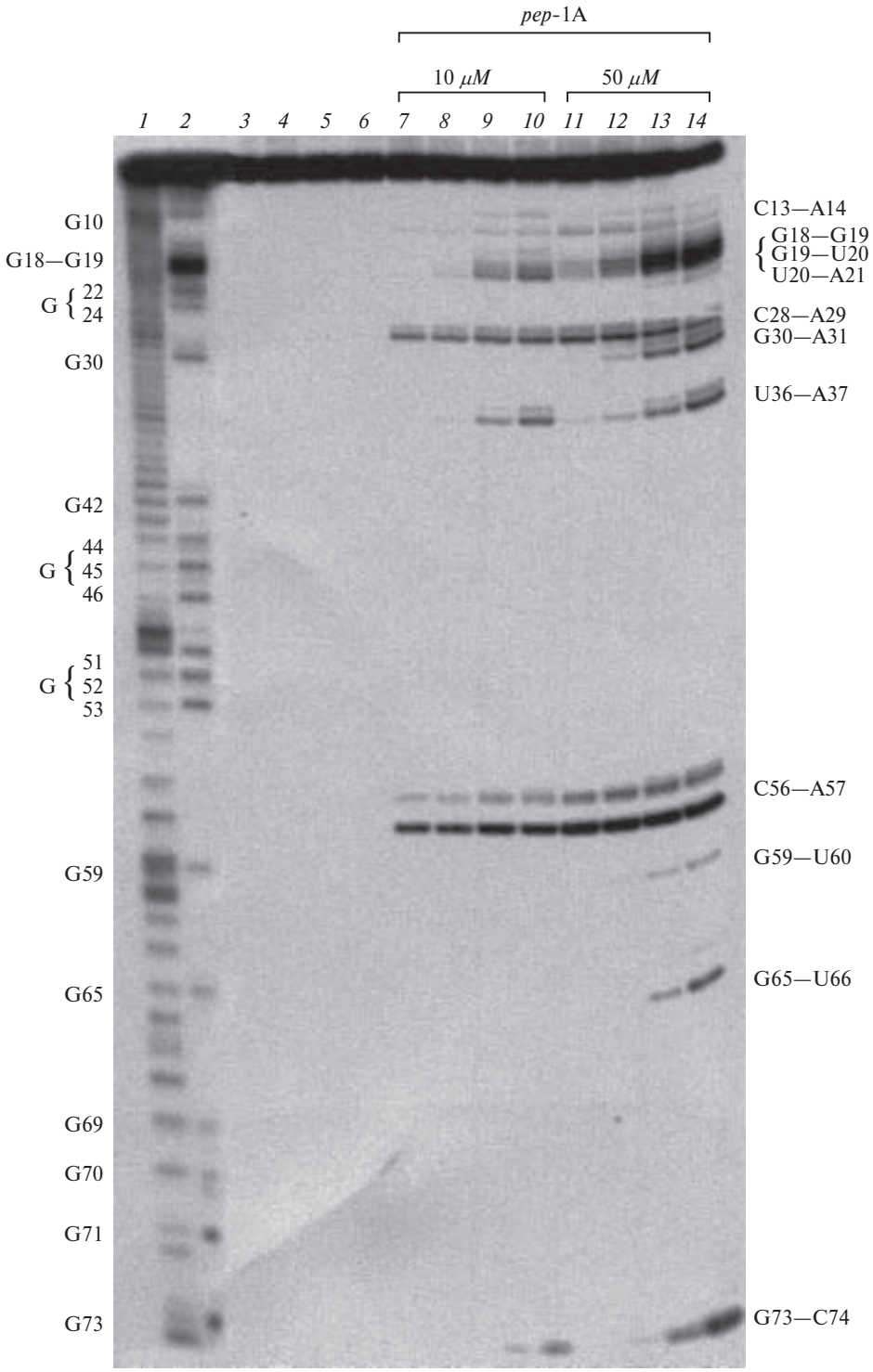



**Fig. 4**. Cleavage of the 3′-[$^{32}$P]-labeled *in vitro* transcript of $tRNA^{Lys}_3$ by the conjugate *pep*-1A, analysis in denaturing 12% PAAG: *1* and *2*, hydrolysis of $tRNA^{Lys}_3$ with 2 *M* imidazole, pH 7.0, and RNase T1 under the denaturing conditions, respectively; *3* and *4*, controls, incubation of $tRNA^{Lys}_3$ in the absence of the conjugate for 1 and 24 h, respectively; *5*, incubation of $tRNA^{Lys}_3$ in the presence of 1 μ*M* peptide $[ArgLeu]_4$ for 24 h; *6*, incubation of $tRNA^{Lys}_3$ in the presence of 1 μ*M* oligonucleotide 1A for 24 h; *7*, *8*, *9*, and *10*, incubation of $tRNA^{Lys}_3$ in the presence of 10 μ*M* conjugate *pep*-1A at 37 °C for 3, 6, 18, and 42 h, respectively; *11*, *12*, *13*, and *14*, incubation of $tRNA^{Lys}_3$ in the presence of 50 μ*M* conjugate *pep*-1A at 37 °C for 3, 6, 18, and 42 h, respectively. The sites of hydrolysis by RNase T1 and the conjugate *pep*-1A are given to the left and right of the figure, respectively.

pin, at the U36—A37, G30—A31, and C28—A29 bonds in the anticodon hairpin, and at the U20—A21, G19—U20, G18—G19, G15—U16, C13—A14, and U8—A9 bonds in the D loop of the *in vitro* transcript of $tRNA^{Lys}_3$. It can be assumed that hydrolysis at these sites occurs due to the formation of imperfect complexes whose accumulation is increased as the concentration of the conjugate is increased (see Fig. 2). It should be noted that the extent of hydrolysis at the C56—A57 bond, which takes place in the full-sized complex, is somewhat increased as the concentration of the reagent is increased from 5 to 50 μmol $L^{-1}$ although this complex exists throughout the entire concentration range.

The plots of the extent of $tRNA^{Lys}_3$ cleavage by the conjugate *pep*-1A *vs.* the time at different concentrations of the conjugate are shown in Fig. 5. After incubation for 42 h, the total extent of RNA hydrolysis at all sites reached 8% (5 *μM pep*-1A, curve *3*), 17% (10 *μM pep*-1A, curve *2*), and 57±5% (50 *μM pep*-1A, curve *1*). The data on the accumulation of the products of $tRNA^{Lys}_3$ hydrolysis at different phosphodiester bonds are presented in Fig. 6. It can be seen that the kinetic curves for the accumulation of hydrolysis products differ in character. Hydrolysis of tRNA at the C56—A57 phosphodiester bond, *i.e.*, in the vicinity of the full-sized hybrid complex, proceeded rapidly. Thus a noticeable extent of hydrolysis was observed already after incubation for 3 h, and the curve flattened out (15%) after 18 h. This type of the kinetic curve (saturated curve) is characteristic of the reactions proceeding in stable complementary complexes.[27] By contrast, the accumulation of products of $tRNA^{Lys}_3$ hydrolysis at other phosphodiester bonds, *viz.*, at G18—G19, G19—U20, and U20—A21 in the D loop (22%), at G73—C74 (12%) at the basis of the amino-acceptor stem, and at G30—A31 and U38—A37 (7 and 3%, respectively) in the anticodon hairpin, depends linearly on the time up to 42 h. This character of the kinetic

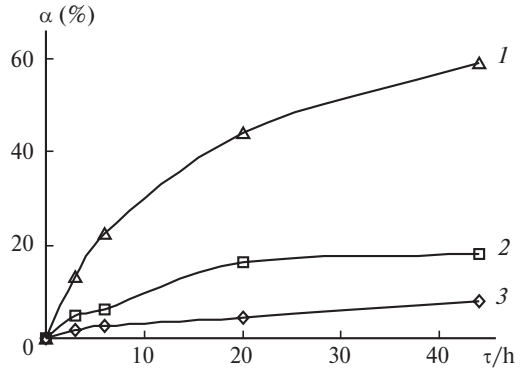



**Fig. 5.** Kinetics of cleavage of the *in vitro* transcript of $tRNA^{Lys}_3$ by the conjugate *pep*-1A. Incubation of $tRNA^{Lys}_3$ in a standard buffer at 37 °C the presence of the conjugate *pep*-1A. The concentration of the conjugate *pep*-1A (μmol $L^{-1}$): *1*, 50; *2*, 10; *3*, 5.

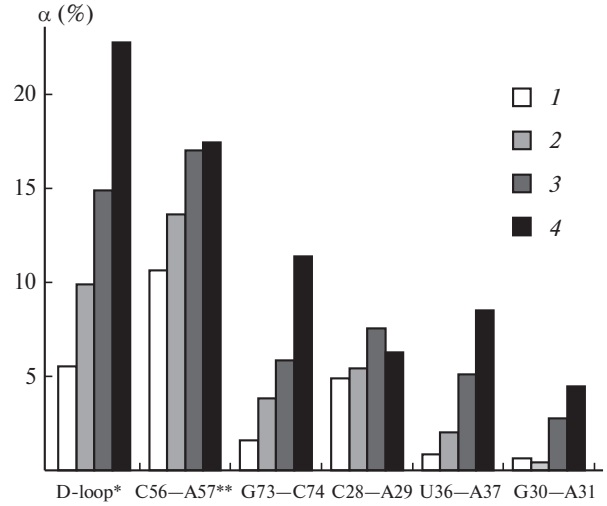



**Fig. 6.** Kinetics of cleavage of the *in vitro* transcript of $tRNA^{Lys}_3$ by the conjugate *pep*-1A at different sites after incubation for 3 (*1*), 6 (*2*), 18 (*3*), and 42 h (*4*).
* The overall extent of depolymerization of $tRNA^{Lys}_3$ at the G18—G19, G19—U20, and U20—A21 phosphodiester bonds in the D loop.
** The phosphodiester bond in the vicinity of the site of binding of the conjugate *pep*-1A.

curves suggests that cleavage at these sites occurs either within imperfect complexes or under the action of the conjugate present in solution. Both pathways are theoretically possible at a high concentration of the conjugate *pep*-1A (50 μmol $L^{-1}$). However, considering that the targeting of tRNA cleavage is a non-random event and the efficiency of hydrolysis at these sites (Fig. 5) as well as the portion of imperfect complexes is increased as the concentration of the reagent is increased (see Fig. 2), it is more probable that the reaction occurs within these imperfect complexes.

The fact that the RNA cleavage at the C56—A57 bond is a complementary addressed process is supported by suppression of the conjugate activity with an excess of the non-modified oligonucleotide. Cleavage of $tRNA^{Lys}_3$ by the conjugate *pep*-1A (10 μmol $L^{-1}$) in the presence of a fivefold excess of the oligonucleotide 1A (50 μmol $L^{-1}$), which is a competitive inhibitor of reactions proceeding within complementary complexes, indicates that the reaction at the C56—A57 bond is inhibited to the highest extent (by a factor of 1.8). For the G18—G19, G19—U20, U20—A21, C28—A29, U36—A37, and G73—C74 bonds, the reaction was suppressed by only 10—20%. This is an argument in support of the assumption that the cleavage at these sites proceeds within imperfect complexes. A slight decrease in the extent of hydrolysis in the presence of an excess of the oligonucleotide 1A may be associated with the high exchange rate of unstable complexes.

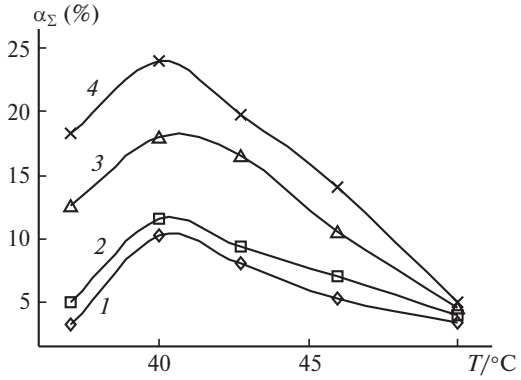



**Fig. 7.** Plots of the extent of cleavage ($\alpha_\Sigma$) of the *in vitro* transcript of $tRNA^{Lys}_3$ by the conjugate *pep*-1A *vs.* the temperature for different incubation times: 3 (*1*); 5 (*2*); 10 (*3*); 24 h (*4*). [3′-$^{32}$P]—$tRNA^{Lys}_3$ was incubated under the standard conditions in the presence of 10 $\mu M$ conjugate *pep*-1A.

The temperature dependence of $tRNA^{Lys}_3$ cleavage by the conjugate *pep*-1A is shown in Fig. 7. In the temperature range from 37 to 50 °C, the efficacy of RNA cleavage is initially increased (up to 40 °C) and then the extent of tRNA hydrolysis is gradually decreased. At 50 °C, the cleavage occurs as a background process, *i.e.*, it is time independent and amounts to 3—5%. The temperature dependence of the extent of $tRNA^{Lys}_3$ cleavage by the conjugate *pep*-1A is bell-shaped with a maximum at 40 °C, which is attributable to the fact that the reaction occurs within both a perfect complex possessing a high melting point and a series of unstable imperfect complexes.

The analysis of the RNA-hydrolyzing activity of the conjugate *pep*-1A and its binding to $tRNA^{Lys}_3$ revealed a number of characteristic features. The conjugate *pep*-1A is efficiently invaded into the $tRNA^{Lys}_3$ structure. At micromolar concentrations of *pep*-1A, $tRNA^{Lys}_3$ is quantitatively involved in the complex, the formation of imperfect complexes being observed along with the formation of a perfect complex. The formation of several imperfect complexes is confirmed by the appearance of a diffusion band in the electrophoretic analysis under the native conditions, the data of the $tRNA^{Lys}_3$ structure probing in the complex with the oligonucleotide 1A, and the character of tRNA cleavage with the conjugate *pep*-1A. The probable schemes of the formation of complexes through incomplete pairing of the conjugates with $tRNA^{Lys}_3$ were revealed by the context analysis.

The maximum rate of RNA cleavage was achieved when the concentration of the conjugate was an order of magnitude higher than that sufficient for the formation of the complex with RNA. A comparison of the data on the rate of RNA hydrolysis at the C56—A57 bond depending on the concentration of the conjugate with the data on binding of the conjugate to RNA leads to the conclusion that the conjugate exists in forms, which are either active or inactive in the cleavage of the phosphodiester bonds in RNA. Within the stable 16-mer heteroduplex with $tRNA^{Lys}_3$, the active *pep*-1A molecules bound to RNA rapidly hydrolyze the latter. In complexes containing the inactive form of the conjugate, the reaction does not take place, and the replacement of inactive molecules in the complex by active molecules from the solution proceeds slowly due to slow dissociation of the duplex. As a result, the kinetic curve for $tRNA^{Lys}_3$ hydrolysis at the C56—A57 bond reaches a plateau when the extent of cleavage reaches ~17% (see Fig. 6). Hydrolysis of RNA by active molecules involved in unstable imperfect complexes proceeds more slowly, but this reaction is accompanied by the rapid replacement of the conjugate molecules bound to RNA with the conjugate molecules from the solution. Due to the rapid exchange, active molecules from the solution can easily reach the regions of imperfect hybridization resulting in the efficient cleavage in these sites. Analogous observations were made in the studies of alkylation of DNA with oligonucleotide derivatives[29] and chemical ligation.[30] These experiments showed that in some cases the reactions proceed more efficiently within unstable complexes in which rapid exchange can take place.

Based on the above-considered results, the following conclusions can be made regarding the size of the addressing region of oligonucleotide conjugates bearing catalytic groups. To ensure the catalytic mode of the reaction, the size of the oligonucleotide fragment of the conjugate must be sufficiently short to provide the dynamic equilibrium between the RNA-bound and free conjugates. The minimal length of the construction is limited by the size necessary for the interaction with the target sequence. In the case of highly structured regions of targets, which necessitate the use of long oligonucleotide addresses, the reaction can be substantially inhibited due to slow exchange of the molecules in the complex. This poses yet another problem, *viz.*, the formation of tandem complexes of conjugates with RNA. In this connection, the use of targets with sequences involved in structures with short stable hairpins, such as tRNA, is non-optimal.

The results obtained in the present study account for low efficacy of the reactions of oligonucleotide conjugates observed in many investigations where RNA-hydrolyzing constructions were covalently linked to oligonucleotides that form stable complexes with an RNA target. These results also give researchers a new line of investigation aimed at the design of highly specific and efficient artificial ribonucleases operating in the catalytic manner.

This study was supported by the Russian Foundation for Basic Research (Project Nos. 99-04-49538,

99-04-49731, and 00-15-97969), the INTAS (Grant 96-1418), the Wellcome Trust (Grant 063630), the US Civilian Research and Development Foundation (CRDF, Grant REC-008), and the Siberian Branch of the Russian Academy of Sciences (Grant 26 and Grant for Young Scientists).

## References

1. J. Hovinen, A. Guzaev, A. Azhayev, and H. Lönnberg, *J. Org. Chem.,* 1995, **60**, 2205.
2. M. A. Reynolds, T. A. Beck, and L. J. Arnold, *Nucleic Acids Res.*, 1996, **24**, 760.
3. K. Ushijima and H. Takaku, *Biochim. Biophys. Acta*, 1998, **1379**, 217.
4. V. Silnikov, G. Zuber, J.-P. Behr, R. Giege, and V. Vlassov, *Phosphorus*, *Sulfur*, *Silicon Relat. Elem.*, 1996, **109—110**, 277.
5. M. Komiyama and T. Inokawa, *J. Biochemistry*, 1994, **116**, 719.
6. J. K. Bashkin, E. I. Frolova, and U. S. Sampath, *J. Am. Chem. Soc.*, 1994, **116**, 5981.
7. V. M. Belkov, N. F. Krynetskaya, E. M. Volkov, Z. A. Shabarova, N. Yu. Krainova, G. N. Novodarova, and M. E. Vol´pin, *Bioorg. Khim.*, 1995, **21**, 446 [*Russ. J. Bioorg. Chem.*, 1995, **21** (Engl. Transl.)].
8. S. Matsuda, A. Ishikubo, A. Kuzuya, M. Yashiro, and M. Komiyama, *Angew. Chem., Int. Ed.*, 1998, **37**, 3284.
9. D. Magda, M. Wright, S. Crofts, A. Lin, and J. L. Sessler, *J. Am. Chem. Soc.*, 1997, **119**, 6947.
10. D. Magda, S. Crofts, A. Lin, D. Miles, M. Wright, and J. L. Sessler, *J. Am. Chem. Soc.*, 1997, **119**, 2293.
11. J. Hall, D. Husken, and R. Haner, *Nucleic Acids Res.*, 1996, **24**, 3522.
12. J. Hall, D. Husken, U. Pieles, H. Mozer, and R. Haner, *Chem. Biol.*, 1994, **1**, 185.
13. V. Vlassov, T. Abramova, R. Giege, and V. Silnikov, *Antisense Nucleic Acid Drug Dev.*, 1997, **7**, 39.
14. N. G. Beloglazova, N. N. Polushin, V. N. Silnikov, M. A. Zenkova, and V. V. Vlassov, *Dokl. Akad. Nauk,* 1999, **369**, 827 [*Dokl. Chem.*, 1999 (Engl. Transl.)].
15. N. G. Beloglazova, V. N. Silnikov, M. A. Zenkova, and V. V. Vlassov, *FEBS Lett.*, 2000, **481**, 277.
16. L. Yurchenko, V. Silnikov, T. Godovikova, G. Shishkin, J.-J. Toulme, and V. Vlassov, *Nucleosides, Nucleotides, Nucleic Acids*, 1997, **16**, 1741.
17. W. F. Lima and S. T. Crook, *International Conference Antisense 98 "Targeting the Molecular Basis of Disease" (London, October 8—9, 1998)*, London, 1998.
18. B. Barbier and A. Brack, *J. Am. Chem. Soc.*, 1988, **110**, 6880.
19. B. Barbier and A. Brack, *J. Am. Chem. Soc.*, 1992, **114**, 3511.
20. D. V. Pyshnyi, M. N. Repkova, S. G. Lokhov, E. M. Ivanova, A. G. Venyaminova, and V. F. Zarytova, *Bioorg. Khim.*, 1997, **23**, 497 [*Russ. J. Bioorg. Chem.*, 1997, **23** (Engl. Transl.)].
21. V. F. Zarytova, E. M. Ivanova, S. N. Yarmolyuk, and I. V. Alekseeva, *Biopolimery Kletka* [*Biopolym. Cell*], 1988, **1**, 220 (in Russian).
22. J. Rudinger, C. Florentz, and R. Giege, *Nucleic Acids Res.*, 1994, **22**, 5031.
23. A. V. Vlassov, V. V. Vlassov, and R. Giege, *Dokl. Akad. Nauk SSSR,* 1996, **349**, 411 [*Dokl. Chem.*, 1996 (Engl. Transl.)].
24. S. Schäfer, H. Heumann, and H. J. Gross, *Nucleic Acids Symposium Series*, **37**, 291.
25. K. Isel, Ch. Ehresmann, G. Keith, B. Ehresmann, and R. Marquet, *J. Mol. Biol.*, 1995, **274**, 236.
26. V. A. Petyuk, M. A. Zenkova, R. Giege, and V. V. Vlassov, *FEBS Lett.*, 1999, **444**, 217.
27. V. A. Petyuk, M. A. Zenkova, and V. V. Vlassov, *Proc. II Int. Conf. on Bioinformatics of Genome Regulation and Structure (Novosibirsk, August 7—11, 2000)*, **3**, 16.
28. S. Shibahara, S. Mukai, T. Nishihara, H. Inoue, E. Ohtsuka, and H. Morisawa, *Nucleic Acids Res.*, 1987, **15**, 4403.
29. D. V. Pyshnyi, S. G. Lokhov, E. M. Ivanova, and V. F. Zarytova, *Bioorg. Khim.*, 1998, **24**, 201 [*Russ. J. Bioorg. Chem.*, 1998, **24** (Engl. Transl.)].
30. N. G. Dolinnaya, A. V. Tsytovich, V. N. Sergeev, T. S. Oretskaya, and Z. A. Shabarova, *Nucleic Acids Res.*, 1991, **19**, 3073.

*Received September 25, 2001;*
*in revised form December 24, 2001*