Cleavage of RNA bulge loops by artificial RNases

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Sensitivity of phosphodiester bonds in RNA bulge loops to cleavage by short cationic peptides and compounds based on 1,4-diazabicyclo[2.2.2]octane and its conjugates with imidazole was studied. Bulge loops containing from one to seven nucleotides were formed in RNA upon its hybridization with partially complementary oligodeoxyribonucleotides. The efficiency of RNA cleavage depends on the length of a bulge loop, the position of the cleaved phosphodiester bond in the loop, and the nature of the RNA-binding fragment of chemical ribonuclease (1,4-diazabicyclo[2.2.2]octane or a cationic peptide). In the absence of Mg^{2+} ions, the phosphodiester bond in the CA motif located in the apical position in 4-, 6-, or 7-membered loops is cleaved with the highest efficiency. In the presence of magnesium ions, the selectivity of RNA cleavage within bulge loops is substantially enhanced. In the case of 1,4-diazabicyclo[2.2.2]octane-based compounds, RNA is subjected to cleavage predominantly at the bonds in 4-, 6-, and 7-membered loops, whereas cleavage of other bonds is greatly suppressed.

Key words: site-selective RNA cleavage, RNA bulge loops, artificial ribonucleases; RNA:oligonucleotide complexes.

In recent years, numerous low-molecular-weight compounds capable of cleaving RNA under physiological conditions have been designed.[1—4] The RNA cleavage by these compounds occurs predominantly at the regions that are not involved in double-stranded structures, such as various loops, single-stranded regions, and junctions. The influence of the RNA sequence and secondary structure on the efficiency and specificity of cleavage was examined only for divalent metal ions.[5—9] The cleavage of phosphodiester bonds located in hairpin loops was studied in detail[5—7] since a hairpin is the main structural element of natural RNA and because of important biological functions of hairpin loops.[10—12] Among various RNA loops, bulge loops, *i.e.*, RNA regions containing nucleotides that have no complementary bases in the opposite RNA strand are of special interest. These loops arrange the RNA tertiary structure,[13,14] serve as specific RNA-protein interaction sites,[15] and form ribozyme active sites.[16]

RNA bulge loops are sensitive to cleavage by a wide range of compounds and are potential targets for antisense oligonucleotide-based conjugates exhibiting site-selective cleavage of RNA. Phosphodiester bonds within RNA bulge loops display a high sensitivity to cleavage by metal ions.[5,8,9] Bonds in most of RNA bulge loops or RNA:DNA complexes are cleaved by divalent metal ions, the bonds located at the 3´-side (in some cases, at the 5´-side) of each nucleotide in the loop being predominantly cleaved.[5] In addition, bonds that are adjacent to the bulge loop but are involved in an RNA:DNA duplex were subject to cleavage.[5]

Earlier,[17] we have studied the sensitivity of phosphodiester bonds in one-to-seven-membered RNA bulge loops to cleavage by the 1,4-diazabicyclo[2.2.2]octane—histidine conjugate (compound ABL4C3), which cleaves RNA selectively at bonds in 4- and 7-membered loops in the presence of magnesium ions. In the present study, we carried out a comparative study of the sensitivity of bonds in RNA bulge loops to cleavage by short cationic peptides mimicking the active center of RNase A[18,19] and by 1,4-diazabicyclo[2.2.2]octane-based compounds bearing oligomethylene fragments at the quaternized nitrogen atoms.[20]

Experimental

Deoxyribonucleoside triphosphates, ribonucleoside triphosphates, acrylamide, *N*,*N*´-methylenebisacrylamide, $LiClO_4$, dithiothreitol (DTT), $MgCl_2$, T4 polynucleotide kinase, RNase T1, imidazole, EDTA, Tris (Sigma, USA), [γ^{32}P]ATP, and [5´-^{32}P]pCp with a specific activity of ~4000 Ci mmol^{-1} (Biosan, Russia) were used; T7 phage RNA polymerase was provided by V. N. Ankilova (Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences). Artificial ribonucleases used in the present study were synthesized in the Laboratory of Organic Synthesis (this Institute). All buffer solutions and reaction samples were

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Table 1. Oligonucleotides used for the formation of artificial bulge loops in M2-96 RNA

Oligonucleotide	Oligonucleotide sequence* (5′ → 3′)	Position in M2-96 RNA	Position of the loop in M2-96 RNA
ON1(0)	GCACTCTGCTGTTCCT	50—65	—
ON2(1/–)	CACTCTGC#GTTCCTTTC	47—64	56
ON3(1/+)	CACTCTGC#AGTTCCTTTC	47—64	56
ON4(1/–)	CAGCACTC#GCTGTTCC	51—67	59
ON5(2/–)	CACTCTGC#TTCCTTTCG	46—64	55—56
ON6(2/+)	CACTCTGC#ATTCCTTTCG	46—64	55—56
ON7(2/–)	CAGCACTC#CTGTTCCTT	49—67	58—59
ON8(3/–)	CACTCTGC#TCCTTTCGA	45—64	54—56
ON9(3/+)	CACTCTGC#ATCCTTTCGA	45—64	54—56
ON10(3/–)	AGCACTC#TGTTCCTTT	48—66	57—59
ON11(4/–)	GCACTCTG#TCCTTTCGA	45—65	54—57
ON12(4/+)	GCACTCTG#ATCCTTTCGA	45—65	54—57
ON13(4/–)	AGCACTCT#TTCCTTTCG	46—66	55—58
ON14(4/–)	CAGCACTC#GTTCCTTTC	48—68	54—57
ON15(5/–)	AGCACTCT#TCCTTTCG	45—66	54—58
ON16(5/+)	AGCACTCT#ATCCTTTCG	45—66	54—58
ON17(5/–)	ACAGCAC#TGTTCCTTT	48—68	57—61
ON18(6/–)	CAGCACTC#TCCTTTCGA	45—67	54—59
ON19(6/+)	CAGCACTC#ATCCTTTCGA	45—67	54—59
ON20(6/–)	ACAGCACT#TTCCTTTCG	46—68	55—60
ON21(7/–)	CACAGCAC#TTCCTTTCG	45—68	54—60
ON22(7/+)	CACAGCACT#ATCCTTTCGA	45—68	54—60
ON23(7/–)	CACAGCAC#TTCCTTTCG	46—69	55—61
ON24(7/–)	CAGCACTC#CCTTTCGAT	44—69	53—59

* A is an additional noncomplementary adenosine opposite the bulge loop.
# The position of the loop in M2-96 RNA.

prepared using MilliQ water (Millipore, USA). All buffers and solutions of artificial RNases were sterilized by filtration through a nitrocellulose filter (0.22 μm) (Millipore, USA). Polyacrylamide gels (PAAG) were analyzed on a Molecular Imager phosphorimager (Bio-Rad, USA).

Oligodeoxyribonucleotides (hereinafter, ON) (Table 1) were synthesized by a standard phosphoramidite method and isolated by reversed-phase HPLC in the Laboratory of Oligonucleotide synthesis (this Institute).

The *in vitro* synthesis of M2-96 RNA (the 96-mer RNA fragment of influenza virus M2 protein mRNA) was performed with the use of DNA-dependent T7 RNA polymerase as described earlier.[21] The 121-mer PCR product, which was prepared by amplification of the pSVK3M2 plasmid with specific primers, was used as a DNA template.[17] The [^{32}P] label was introduced at the 5′ terminus of RNA according to a standard method.[22]

Cleavage of M2-96 RNA in complexes with oligodeoxyribonucleotides by chemical ribonucleases

Before cleavage, M2-96 RNA was hybridized with an oligonucleotide. For this purpose, the reaction mixture (10 μL), which contained [^{32}P]-labeled M2-96 RNA (10000 cpm Cherenkov's counting), a 50 *mM* Tris-HCl buffer, pH 7.0, 0.2 *M* KCl, 0.5 *mM* EDTA, 100 μg mL^{-1} total *E. coli* tRNA, and one of oligodeoxyribonucleotides at a concentration of 10^{-6} mol L^{-1}, was incubated at 37 °C for 1 h. Then one of artificial ribonucleases (KHR, 2L2, Dp12, or ABL4C3) at concentrations ranging from 10^{-5} to 10^{-3} mol L^{-1} (the concentrations of the compounds are given in the figure captions) was added, and the resulting mixture was incubated at 37 °C for 4—24 h. The reaction was quenched by precipitation with 2% $LiClO_4$ in acetone. The RNA pellet was separated by centrifugation. The M2-96 RNA cleavage products were analyzed by electrophoresis in 18% PAAG containing 8 *M* urea. The M2-96 RNA cleavage sites were identified by comparing with the data on partial cleavage of the same RNA by RNase T1 under denaturating conditions[22] and in a 2 *M* imidazole buffer, pH 7.0, at 90 °C.[23] The extent of RNA cleavage was estimated as the ratio of the radioactivity in the band corresponding to the RNA cleavage product to the total radioactivity of the sample applied onto the lane.

Cleavage of RNA:oligonucleotide duplexes by RNase A

The M2-96 RNA:ON complexes were prepared as described above. After incubation of RNA with ON for 1 h, RNase A at a concentration of 10^{-5} mg mL^{-1} was added, and the mixture was incubated at 37 °C for 1 min. Then a solution of total *E.coli* tRNA (100 μg mL^{-1}, 40 μL) was added, and the reaction mixture was successively extracted with a 1 : 1 phenol—chloroform mixture and chloroform. Then RNA and its fragments were precipitated with ethanol in the presence of 0.3 *M* sodium acetate, pH 5.2. The cleavage products were analyzed as described above.

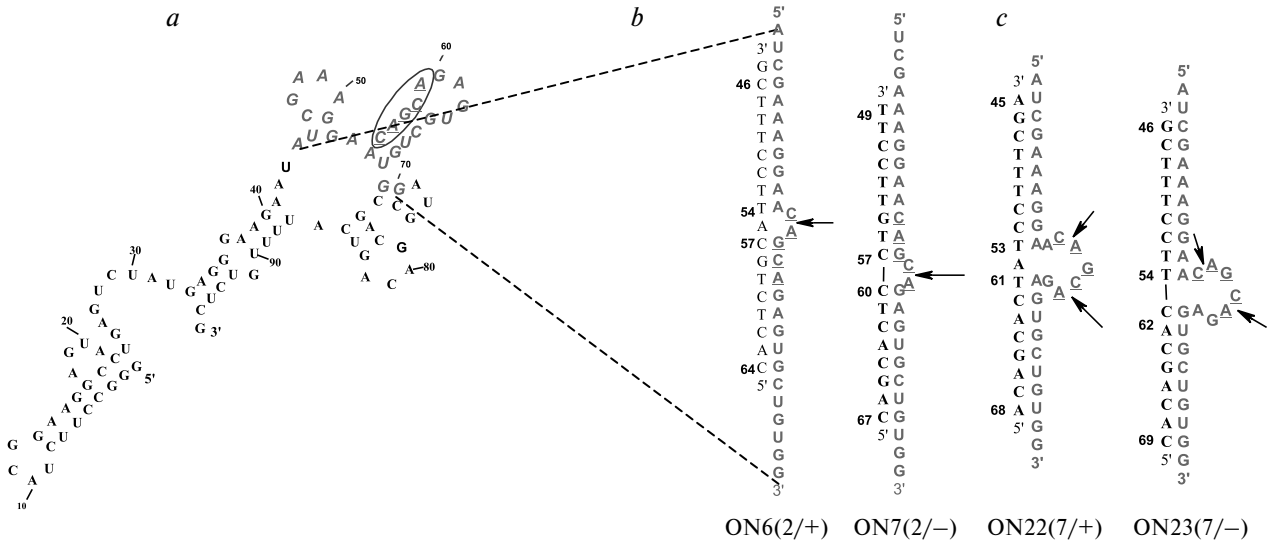


Fig. 1. Secondary structure of M2-96 RNA (*a*) and the structures of the bulge loops containing one of the C—A bonds (*b*) or both C—A bonds (*c*) of the $C^{55}AGCA^{59}$ sequence of M2-96 RNA formed by hybridization of M2-96 RNA with oligodeoxyribonucleotides (ON). The hairpin sequence, to which oligodeoxyribonucleotides are complementary, is shown in gray; the $C^{55}AGCA^{59}$ sequence is indicated by an oval. The numbers of oligodeoxyribonucleotides are given at the bottom of the figure.

Results and Discussion

Model RNA

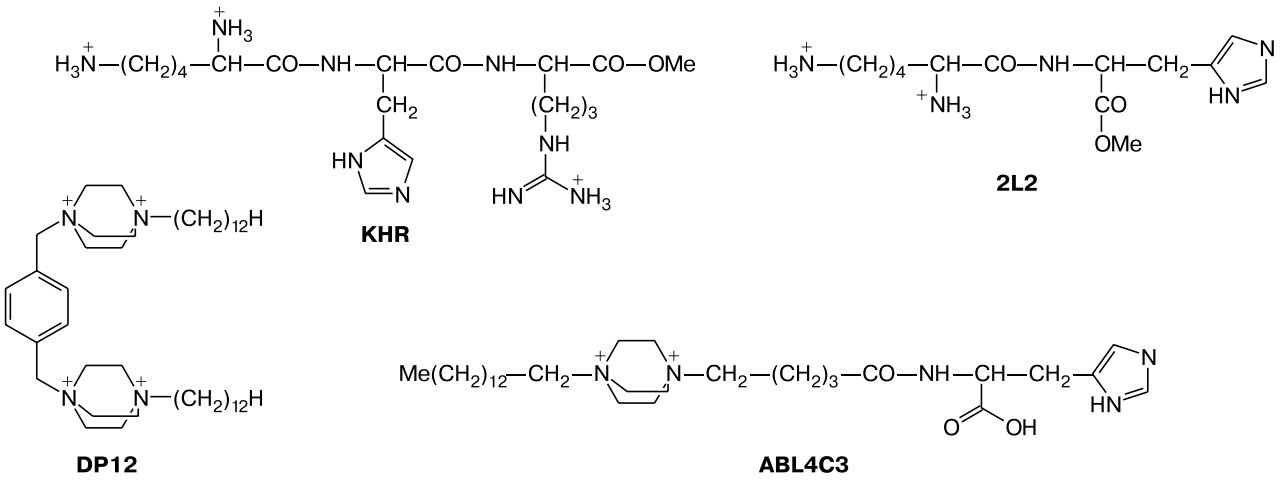
Sensitivity of phosphodiester bonds in various elements of RNA secondary structure to cleavage by chemical ribonucleases was studied in experiments with the 96-mer RNA fragment of influenza virus M2 protein mRNA (hereinafter, M2-96 RNA) (Fig. 1, *a*). This RNA is of interest because M2 RNA encodes M2 membrane protein, which forms ion channels of virions and is highly conserved.[24—26] Due to the role of this protein in the life cycle of the influenza virus and its conservativeness, the RNA encoding this protein is a promising target for the development of antivirals based on oligonucleotide conjugates. The fragment of the influenza virus M2 protein RNA corresponds to the region 202—297. Earlier, it has been demonstrated[27] that the $C^{256}AGCA^{260}$ sequence in the M2 RNA hairpin (corresponding to the $C^{55}AGCA^{59}$ sequence in M2-96 RNA) is cleaved by chemical ribonuclease ABL4C3 to a small degree. It has also been demonstrated[28] that two adjacent Pyr*—A bonds in a single-stranded RNA are very sensitive to various cleaving agents.

Oligonucleotides and RNA-cleaving agents

To construct bulge loops in M2-96 RNA, the latter was hybridized with oligodeoxynucleotides (consisting of 15—18 nucleotides) partially complementary to the RNA (see Fig. 1, *b*, *c* and Table 1). The oligonucleotide sequences were chosen so that they were not self-complementary and both shoulders of the oligonucleotide had similar hybridization properties. The oligonucleotides that form artificial bulge loops upon binding to M2-96 RNA are denoted as ON*m*(*n*/±), where *m* is the number of the oligonucleotide, *n* is the length of the artificial bulge loop formed by the oligonucleotide in M2-96 RNA, and the plus and minus signs indicate respectively the presence or in the absence of noncomplementary adenosine residue in the oligonucleotide opposite the artificial bulge loop.[17] For each loop of a particular length, two or three different sequences were studied: loops consisting of up to four nucleotides comprised either the $C^{55}—A^{56}$ or $C^{58}—A^{59}$ motif (see Fig. 1, *b*); longer loops comprised both these motifs (except for the loop formed by ON17(5/−)) and differed in the location of the $C^{55}ACCA^{59}$ sequence in the loop (see Fig. 1, *c*). The oligonucleotide ON1(0) entirely complementary to the sequence 50—65 in M2-96 RNA was used as the control. The structures of the bulge loops in all M2-96 RNA:ON complexes were studied by gel-shift assay and by probing RNA structure in complexes by RNase A and a 2 *M* imidazole buffer.[17] It was demonstrated that all oligonucleotides at a concentration of 10^{-6} mol L^{-1} are capable to bind efficiently (≥80%) to M2-96 RNA and form bulge loops of different length.

The following most active compounds, which have been studied earlier in our laboratory, were used as cleaving agents: (1) short cationic peptides that mimic the active centers of RNases A and T1, *viz.*, the tripeptide KHR (see Ref. 18) and compound 2L2;[19] and (2) compound Dp12 based on two 1,4-diazabicyclo[2.2.2]octane residues, which are connected through a rigid linker and contain oligomethylene fragments at the quaternized

* Pyr is pyrimidine.



nitrogen atoms.[20] The 1,4-diazabicyclo[2.2.2]octane—histidine conjugate (compound ABL4C3)[29] and RNase A were used as controls.

Phosphodiester bond cleavage in artificial loops

Analysis of the sensitivity of the RNA phosphodiester bonds within artificial loops to various RNA-cleaving agents was performed as follows: [5′-^{32}P]-M2-96 RNA (10^{-7} mol L^{-1}) was incubated with the corresponding oligonucleotide (10^{-6} mol L^{-1}) in a 50 *mM* Tris-HCl buffer, pH 7.0, containing 0.2 *M* KCl, 0.5 *mM* EDTA, and a 0.1 μg μL^{-1} RNA carrier (hereinafter, "standard conditions") for 1 h. Then the corresponding compounds were added to the reaction mixture to the optimal concentration (10^{-3} mol L^{-1} for 2L2 and KHR, 10^{-5} mol L^{-1} for Dp12, and $5 \cdot 10^{-4}$ mol L^{-1} for ABL4C3),[18—20,27] and the reaction mixtures were incubated for 24 h at 37 °C (in the case of 2L2, KHR, and Dp12) or 8 h (in the case of ABL4C3). After incubation, the RNA cleavage products were analyzed by electrophoresis in 18% PAAG under denaturating conditions.

Figure 2 displays the autoradiograph of the gel after separation of the products of the M2-96 RNA:ON complex cleavage by KHR. As can be seen from Fig. 2, in the absence of oligonucleotides, M2-96 RNA is cleaved at the three major sites: U^{20}—A^{21}, U^{30}—A^{31}, and U^{43}—A^{44} (lane K1). The less intense cleavage occurs at the phosphodiester bonds after A^{21}, C^{22}, U^{24}, U^{28}, C^{29}, U^{32}, G^{33}, and C^{46}. A similar cleavage pattern was observed in the case of compounds 2L2, Dp12, and ABL4C3. The sensitivity to cleavage of C^{55}—A^{56} and C^{58}—A^{59} bonds, which are located in the stem of the hairpin in native M2-96 RNA, depends on the nature of the RNA-cleaving agent. In the case of ABL4C3 and KHR, the extent of RNA cleavage at these bonds is 2—5 times lower than the average extent of cleavage at the major sites. In the case of Dp12, the extent of RNA cleavage at these bonds is comparable with the extent of RNA cleavage at U^{20}—A^{21} and U^{30}—A^{31} bonds. Evidently, this is attributed to the higher ability of Dp12 to cleave RNA at the bonds in double-stranded regions.[30] In the presence of the control oligonucleotide ON1(0), which is completely complementary to the sequence 50—65 of M2-96 RNA, the cleavage at the C^{55}—A^{56} and C^{58}—A^{59} bonds is completely inhibited and the new cleavage site U^{78}—G^{79} appears. This suggests that the oligonucleotide forms a stable complex with RNA, which protects the C^{55}—A^{56} and C^{58}—A^{59} bonds from cleavage, and simultaneously unfolds the A^{54}—G^{68} hairpin due to which the U^{78}—G^{79} bond becomes accessible for cleavage.

Compounds KHR, Dp12, and ABL4C3, like RNase A (Fig. 3, *a*), do not cleave bonds in 1-membered bulge loops (M2-96 RNA complexes with the oligonucleotides ON2(1/–), ON3(1/+), and ON4(1/–)). The exception was the M2-96 RNA:ON4(1/–) complex, in which minor cleavage at the C^{58}—A^{59} bond was observed in the presence of Dp12 and ABL4C3 RNA (preliminary results are not reported). The bonds in the bulge loops formed in the presence of all other oligonucleotides are cleaved by both the compounds under study and RNase A. Unlike the cleavage in a 2 *M* imidazole buffer[17] or in the presence of divalent metal ions,[5] no cleavage of other bonds in the bulge (except for C^{55}—A^{56} and C^{58}—A^{59}) and in the vicinity of the bulge was observed (see Figs 2 and 3).

Compound 2L2 does not exhibit enhanced activity with respect to phosphodiester bonds within bulge loops. The extents of M2-96 RNA cleavage by this compound at C^{55}—A^{56} and C^{58}—A^{59} bonds within all RNA:oligonucleotide complexes were nearly the same and did no differ from those observed in the control, *viz.*, free M2-96 RNA.

Efficiency and selectivity of cleavage of M2-96 RNA complexes with oligonucleotides by KHR

Quantitative analysis of the data on the M2-96 RNA:ON complex cleavage by KHR (Fig. 4, *a*) shows

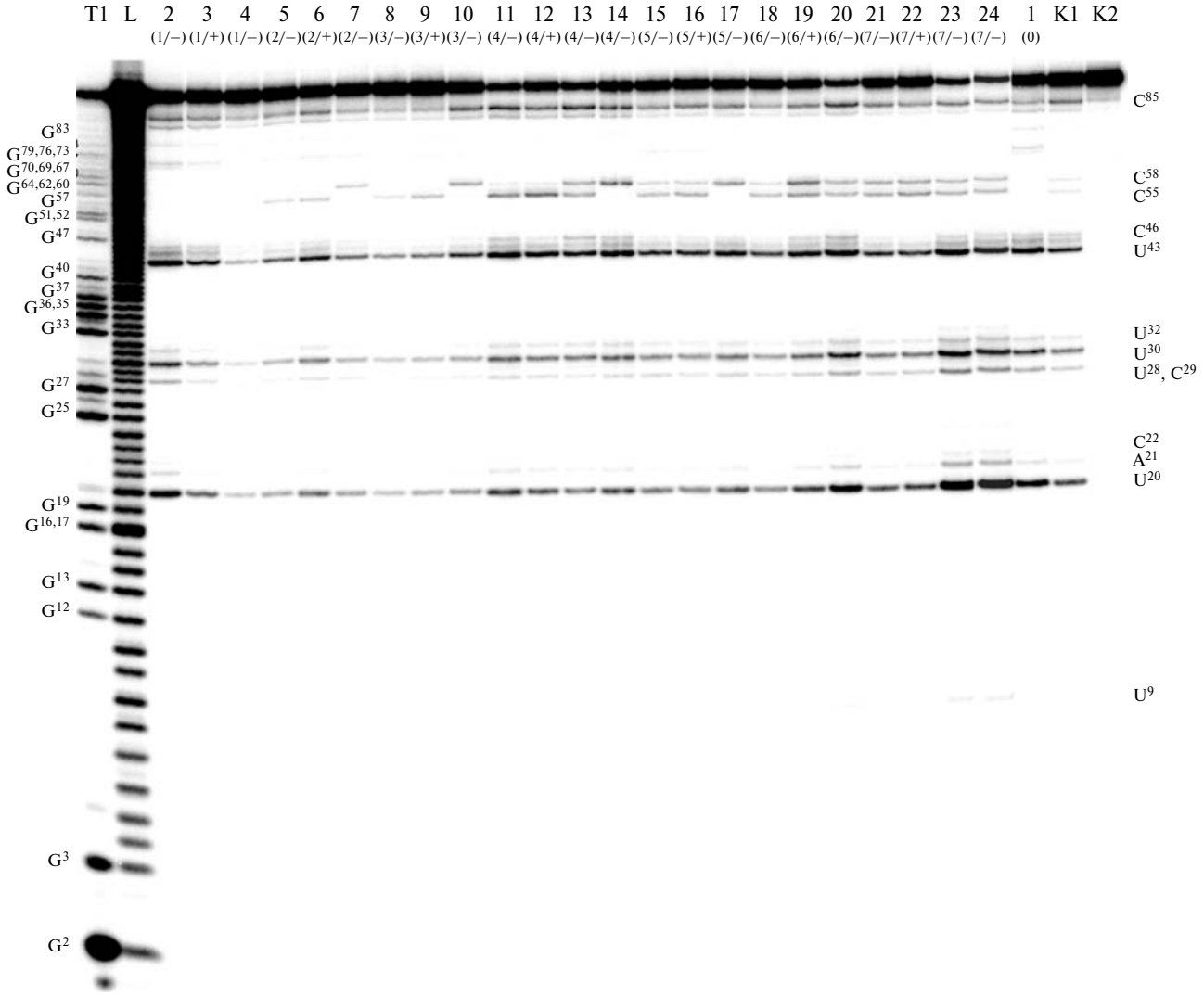


Fig. 2. Cleavage of M2-96 RNA:ON complexes by KHR. The autoradiograph of 18% denaturating PAAG after separation of [5′-^{32}P]-M2-96 RNA cleavage products. M2-96 RNA (10^{-7} mol L^{-1}) was incubated with the corresponding oligonucleotide (10^{-6} mol L^{-1}) under the standard conditions in the presence of KHR (10^{-3} mol L^{-1}) for 24 h at 37 °C. Lane K1, control, M2-96 RNA cleavage in the absence of the oligonucleotide; lane K2, incubation of M2-96 RNA in the absence of KHR. Lanes L and T1, an imidazole ladder and RNA cleavage by RNase T1 under denaturating conditions, respectively. The numbers of oligonucleotides in the presence of which the cleavage was carried out are given over the lanes. The bonds cleaved by RNase T1 and KHR are given to the left and right of the autoradiograph, respectively.

that the total extent of cleavage of the complexes with bulges up to three nucleotides in length is approximately equal to the total extent of cleavage of native M2-96 RNA (20%). The extent of RNA cleavage at bonds within 2- and 3-membered bulges was 0.5—1.5%. An increase in the bulge loop length led to an increase in both the efficiency of bond cleavage in the bulge loop and the total efficiency of M2-96 RNA cleavage. According to calculations, binding of oligonucleotides in the region 45—69 should not change the RNA structure in other parts of the molecule (for example, in the regions U^{20}—A^{21} or U^{30}—A^{31}). However, in the case of the RNA complexes with ON11(4/–), ON14(4/–), ON20(6/–), ON23(7/–), and ON24(7/–), the extent of cleavage at these bonds increases by a factor of 2—4 compared to the control (RNA in the absence of oligonucleotides (K1)). To estimate how much the sensitivity of phosphodiester bonds in artificial bulge loops differs from that of other bonds in M2-96 RNA, we introduced the term "selectivity" as the ratio of the extent of RNA cleavage at the bonds within the bulge loop to the total extent of RNA cleavage in this particular complex. As can be seen from the histogram (see Fig. 4, *b*), the sensitivity of bonds to cleavage increases with increasing length of the bulge. In complexes, in which an oligonucleotide contains noncomplementary adenosine residue opposite the bulge (ON*m*(*n*/+)), the selectivity of

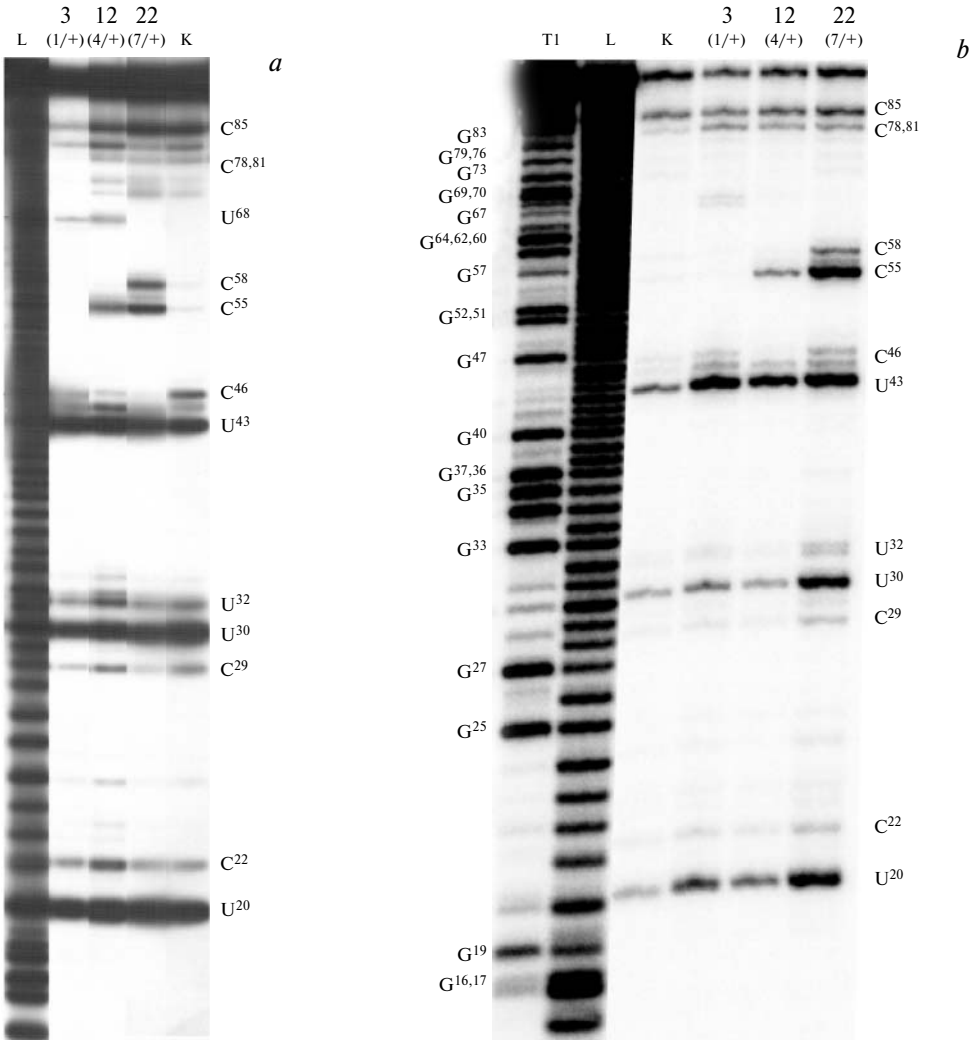


Fig. 3. Cleavage of M2-96 RNA:oligonucleotide complexes by RNase A in the absence (*a*) and in the presence of 10 *mM* $MgCl_2$ (*b*). The autoradiograph of 18% denaturating PAAG after separation of the [5′-^{32}P]-M2-96 RNA cleavage products. M2-96 RNA (10^{-7} mol L^{-1}) was incubated with one of the oligonucleotides ON3(1/+), ON12(4/+), or ON22(7/+) (10^{-6} *M*) under the standard conditions in the presence or in the absence of 10 *mM* $MgCl_2$ and in the presence of RNase A (10^{-5} mg mL^{-1}) at 37 °C for 1 min. Lane K, M2-96 RNA cleavage by RNase A in the absence of oligonucleotides. Lanes L and T1, random RNA cleavage in a 2 *M* imidazole buffer, pH 7.0, and by RNase T1 under denaturating conditions, respectively. The oligonucleotide numbers are shown over the lanes. The bonds cleaved by RNase T1 and RNase A are given to the left and right of the autoradiograph, respectively.

cleavage is always somewhat higher than that in the complexes with the oligonucleotide ON*n*(*m*/–). It should be noted that, according to the results of the study,[17] binding of oligonucleotides containing additional adenosine does not differ from the binding of an oligonucleotide in which noncomplementary adenosine is absent. Consequently, the difference in the selectivity of cleavage in the bulge loop can be attributed to a more flexible conformation of the loop facing the additional adenine, which is favorable for transesterification.

Loops containing up to three nucleotides comprised only one C—A motif. A part of bulge loops consisting of four and five nucleotides also comprised only one C—A bond (ON11(4/–), ON12(4/+), and ON17(5/–)), whereas other bulge loops comprised both C—A bonds (ON13(4/–), ON14(4/–), ON15(5/–), and ON16(5/+)), in the latter case one of the bases in the CAGCA sequence (C or A) being involved in the RNA:DNA duplex. The loops consisting of six and seven nucleotides contained both the C^{55}—A^{56} and C^{58}—A^{59} bonds. The maximum selectivity of cleavage (0.15) of one C—A bond was observed for a 4-membered loop with the C^{55}—A^{56} bond located in the apical position (M2-96 RNA:ON12(4/+) complex). As can be seen from the above data, the presence of the second CA motif in the bulge loop increases the selectivity of cleavage up to 0.21 (the RNA complex with ON19(6/+)). A comparison of the total extents of cleavage of the complexes and the selectivity of RNA

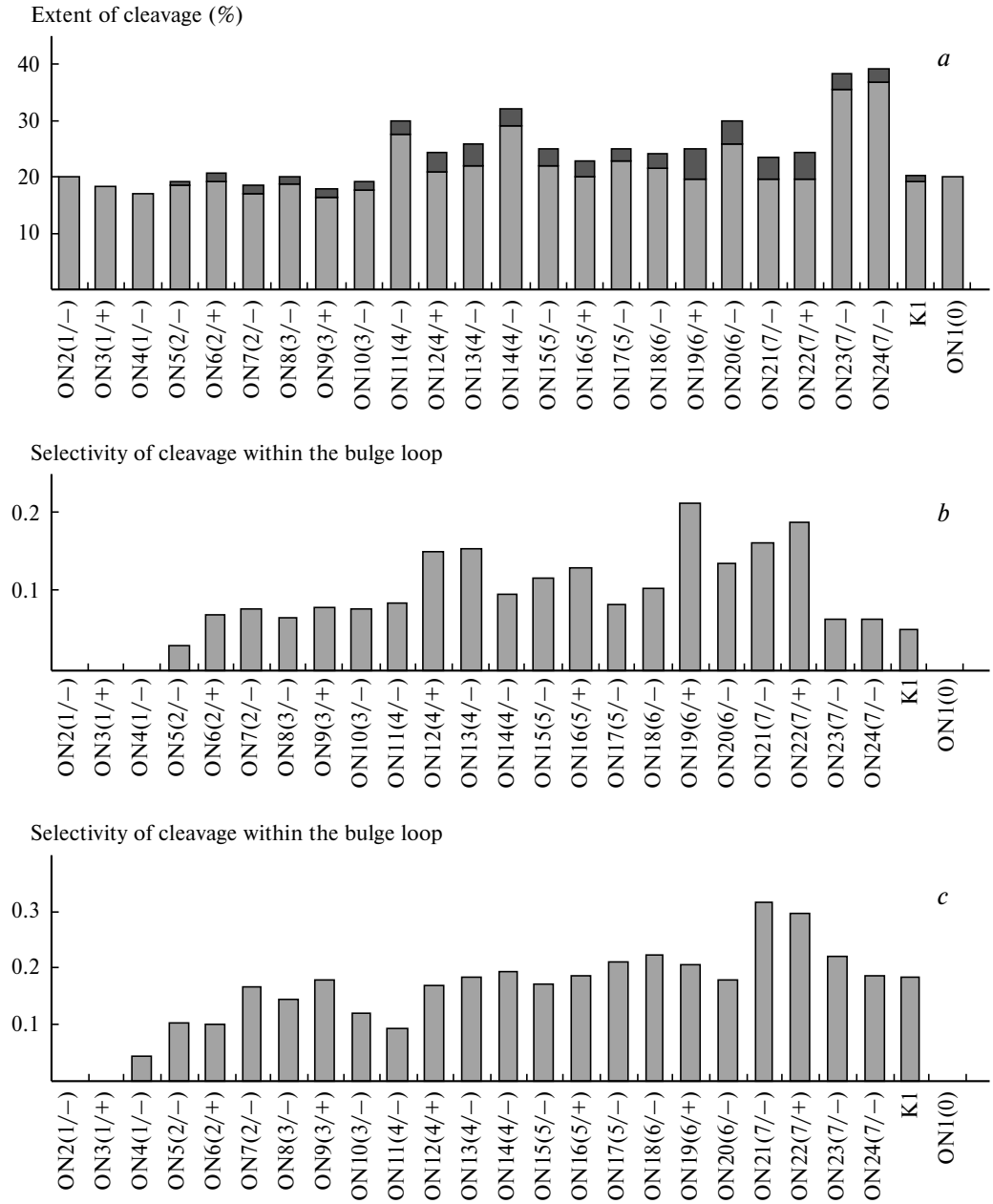


Fig. 4. Quantitative data on M2-96 RNA:oligonucleotide complex cleavage by KHR (*a*, *b*) and Dp12 (*c*). Dark-gray parts of the bars correspond to the extent of cleavage at the bonds within the artificial bulge loop. The selectivity of cleavage (*b*, *c*) was determined as the ratio of the extent of bond cleavage within the artificial bulge loop to the total extent of M2-96 RNA cleavage.

cleavage at the corresponding bulge loops (see Fig. 4, *a*, *b*) showed that the complexes in which the total extent of cleavage (ON23(7/–) and ON24(7/–)) is high are characterized by low selectivity.

Efficiency and selectivity of cleavage of M2-96 RNA complexes with oligonucleotides by compound Dp12

Cleavage of M2-96 RNA by compound Dp12 in the absence of oligonucleotides or in the presence of ON1(0) occurs at the same major sites, as in the case of KHR. A similar RNA cleavage pattern is observed in complexes with oligonucleotides. The total extent of RNA cleavage by Dp12 increases with increasing length of the bulge from 1 to 3 nucleotides, whereas the complexes containing longer bulges are cleaved with approximately the same efficiency. Unlike RNA cleavage by KHR, the maximum extent of RNA cleavage by Dp12 in the RNA:oligonucleotide complexes under the standard conditions is three times higher than the total extent of cleavage of native M2-96 RNA. The extents of RNA cleavage at the

bonds within bulge loops by Dp12 were substantially higher than those achieved in the presence of KHR. Compounds KHR and Dp12 exhibited different selectivities of RNA cleavage (see Fig. 4, *b*, *c*). In the case of Dp12, the selectivity of phosphodiester bond cleavage within 2- and 3-membered loops was lower than that of the cleavage of these bonds in native M2-96 RNA (see Fig. 4, *c*). In the complexes containing 2—5-membered bulge loops, the extent of RNA cleavage at bonds within the loops increases together with an increase in the total extent of RNA cleavage. In longer loops, the extent of bond cleavage within the loop increases, whereas the extent of cleavage of other bonds in RNA decreases. This results in an increase in selectivity of cleavage up to 0.32 (RNA:ON21(7/—) complex). Based on the above results, one can conclude that bonds within bulge loops are more sensitive to cleavage by Dp12 than to cleavage by the tripeptide KHR.

Cleavage of M2-96 RNA:ON complexes by ABL4C3, Dp12, and KHR in the presence of 10 mM $MgCl_2$

It is known[31,32] that Mg^{2+} ions stabilize the RNA structure. An increase in stability of the RNA structure results in a decrease in the efficiency of RNA cleavage by different agents. It was of interest to reveal the influence of magnesium ions on the selectivity of M2-96 RNA cleavage at the bonds within bulge loops.

Analysis of M2-96 RNA cleavage within complexes with oligonucleotides, which form 1-, 4-, or 7-membered bulge loops, by compounds KHR, Dp12, and ABL4C3 and RNase A in the presence and in the absence of 10 *mM* $MgCl_2$ shows that the extent of cleavage of both native M2-96 RNA (see Figs 3 and 5, lanes K) and M2-96 RNA bound with oligonucleotides by all the compounds, as well as by RNase A, decreases in the presence of magnesium ions, which, evidently, reflects stabilization of the M2-96 RNA structure under these conditions. The pattern of M2-96 RNA cleavage by KHR and RNase A remains unchanged in the presence of magnesium ions. In the presence of magnesium ions, a slight increase in the selectivity of RNA cleavage in 4- and 7-membered bulge loops by the short cationic peptide KHR is observed (Fig. 6), whereas the selectivity of M2-96 RNA cleavage by RNase A in the presence of magnesium ions is equal to that in the absence of these ions. Apparently, RNase A and KHR, which mimics the active center of RNase A, exhibit low sensitivity to changes in the RNA structure in the presence of magnesium ions.

For the 1,4-diazabicyclo[2.2.2]octane-based compounds (Dp12 and ABL4C3), the total extent of RNA cleavage in the presence of magnesium ions decreases by a factor of 2—3 compared to that observed under the standard conditions (cleavage in the absence of magnesium ions). In this case, the pattern of M2-96 RNA cleavage also changes (see Fig. 5). In the presence of 10 *mM* $MgCl_2$, cleavage at U^{43}—G^{44} and C^{46}—G^{47} bonds is much weaker than that in the absence of magnesium ions, and the cleavage at U^{20}—A^{21} and U^{30}—A^{31} bonds is completely suppressed. Simultaneously, in the presence of magnesium ions, the extent of RNA cleavage at the bonds within the bulge loops substantially increases. In other words, selective M2-96 RNA cleavage at the bonds in bulge loops is observed. In the M2-96 RNA complexes with the oligonucleotides ON12(4/+) and ON22(7/+), C^{55}—A^{56} and C^{58}—A^{59} bonds in the bulge loops are cleaved by Dp12 with the same or higher efficiency than the most sensitive U^{43}—G^{44} and C^{46}—G^{47} bonds in M2-96 RNA (see Fig. 5). The selectivity of M2-96 RNA cleavage reaches ~0.5 for the 7-membered loop (see Fig. 6, *b*). This selectivity is lower than that observed in the presence of ABL4C3 (0.75).[17] However, an increase in selectivity in the presence of magnesium ions is apparently a general feature of the compounds containing the 1,4-diazabicyclo[2.2.2]octane residue as an RNA-binding group.

Many ribonucleases and ribozymes cleave RNA by a mechanism of intramolecular phosphodiester-bond migration, *i.e.*, transesterification. Transesterification of RNA can occur only if the attacking oxygen atom of the 2′-hydroxy group of the nucleophile is in line with the 5′-oxy anion of the leaving group, *i.e.*, the leaving group (oxygen of the 5′-hydroxy group), the phosphorus atom, and the nucleophile (oxygen of the 2′-hydroxy group) are strictly in-line.[33] Therefore, the sensitivity of phosphodiester bonds in RNA bulge loops to cleavage through the transesterification mechanism is apparently determined by their ability to adopt an in-line conformation. The phosphodiester bond can spontaneously adopt an in-line conformation if the corresponding region of the RNA structure is sufficiently flexible. In the case of bulge loops, this is determined by the length of the loop and the efficiency of stacking interactions between nucleotides within the loop and with the nearest neighbors.[34]

According to calculations, nucleotides in RNA bulge loops can adopt two main conformations, *viz.*, can be either looped-out or stacked in a helical region.[35—40] In loops consisting of more than one nucleotide, a large variety of conformations is observed due to formation of a hydrogen bond network and stacking interactions between nucleotides in the loop and between the nucleotides in the loop and flanking base pairs.[41—43] Neither the conformation of nucleotides in a bulge loop nor the orientation of the catalytic group for efficient RNA cleavage at the bonds in loops can be predicted based on calculations by modern methods.

Earlier, we have shown[17] that the characteristic features of cleavage of RNA bulge loops of different length by ABL4C3 are similar to the data on cleavage of bulge loops by Zn^{2+} ions.[9] In both cases, the most efficient

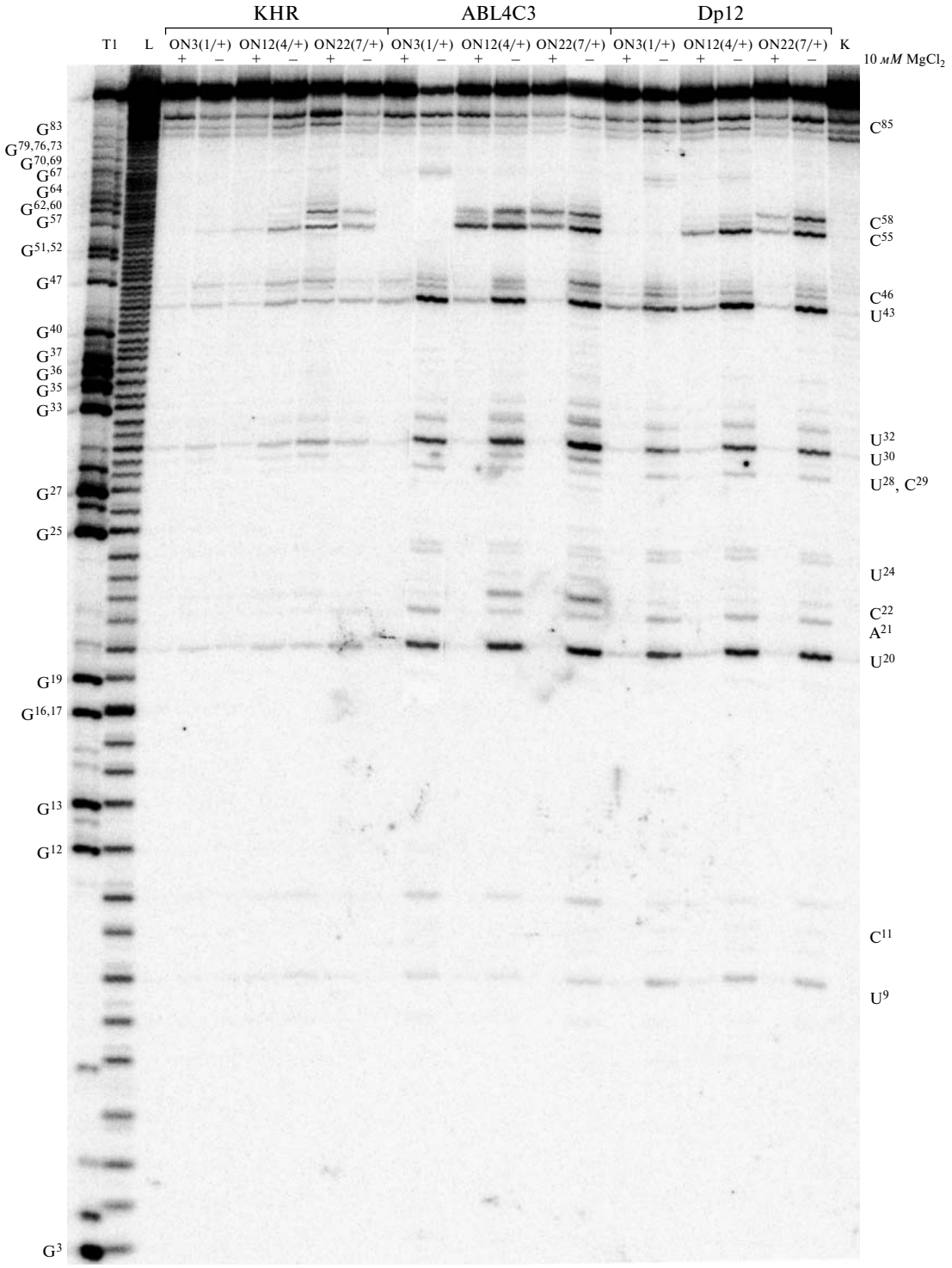


Fig. 5. Comparison of the cleavage of M2-96 RNA:ON complexes by Dp12, KHR, and ABL4C3 in the presence (+) and in the absence (–) of 10 *mM* $MgCl_2$. The autoradiograph of 18% denaturating PAAG after separation of [5′-^{32}P]-M2-96 RNA cleavage products. M2-96 RNA (10^{-7} mol L^{-1}) was incubated with the oligonucleotide ON3(1/+), ON12(4/+), or ON22(7/+) (10^{-6} mol L^{-1}) in a 50 *mM* Tris-HCl buffer, pH 7.0, containing 0.2 *M* KCl, 0.5 *mM* EDTA, and a 0.1 mg mL^{-1} RNA carrier in the presence or in the absence of 10 *mM* $MgCl_2$ and in the presence of Dp12, ABL4C3, or KHR at a concentration of 10^{-5}, $5 \cdot 10^{-4}$, or 10^{-3} mol L^{-1}, respectively, at 37 °C for 8 h (in the case of ABL4C3) or 24 h (in the case of Dp12 and KHR). Lane K, incubation of M2-96 RNA in the absence of the compounds. Lanes L and T1, random RNA cleavage in a 2 *M* imidazole buffer and by RNase T1 under denaturating conditions, respectively. The oligonucleotide numbers are given over the lanes. The bonds cleaved by RNase T1 and the compounds are given to the left and right of the autoradiograph, respectively.

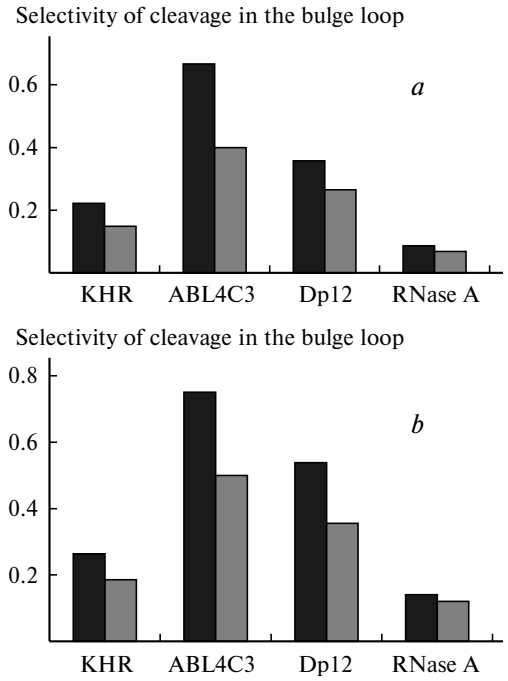


Fig. 6. Selectivity of RNA cleavage at the bonds within the 4-membered (M2-96 RNA:ON12(4/+) complex) (*a*) and 7-membered (M2-96 RNA:ON22(7/+) complex) (*b*) artificial bulge loops in the presence (dark gray bars) and in the absence (light gray bars) of 10 *mM* $MgCl_2$. The selectivity of cleavage was determined as the ratio of the extent of bond cleavage within the artificial bulge loop to the total extent of M2-96 RNA cleavage.

cleavage was observed if the cleaved phosphodiester bond is located in the apical position of the loop consisting of four (for ABL4C3) or five (for Zn^{2+} ions) nucleotides.

The above data demonstrate that artificial ribonucleases and RNase A cleave native M2-96 RNA at the same sites. In general, the pattern of RNA cleavage by artificial RNases of different nature and by RNase A is the same in the case of M2-96 RNA:oligonucleotide complexes containing artificially formed bulge loops as well. However, quantitative differences in the sensitivity of bonds within bulge loops to compounds of different nature are observed. The cationic peptides KHR and 2L2, which mimic the active center of RNase A, as well as natural RNase A, cannot selectively cleave phosphodiester bonds within bulge loops both in the presence and in the absence of magnesium ions. The selectivity of RNA cleavage at the bonds within bulge loops decreases in the series ABL4C3 > Dp12 ≫ KHR ≫ 2L2. These differences can be associated with both weak affinity of cationic peptides for RNA and the fact that cleavage of RNA, which have a pronounced three-dimensional structure, is determining by the efficiency of fitting of the RNA-binding fragment of chemical ribonuclease to the structure of RNA. Compounds containing the 1,4-diazabicyclo[2.2.2]octane fragment (Dp12 and ABL4C3) more efficiently cleave bonds within bulge loops compared to other bonds in RNA, particularly, in the presence of magnesium ions. Apparently, binding of the positively charged 1,4-diazabicyclo[2.2.2]octane residues to internucleotide phosphates depends much stronger on the conformation of the phosphodiester bond than binding of the amino-acid residues (Lys and Arg). It cannot be ruled out that efficient cleavage of bonds in bulge loops by these compounds is a consequence of their higher conformational flexibility compared to other bonds in RNA.

To summarize, we demonstrated that the sensitivity of bonds in RNA to cleavage can be changed. The cleavage rate of particular RNA sequences can be substantially increased by constructing regions with a stressed structure, *viz.*, artificial bulge loops, in RNA. As expected, phosphodiester bonds in such bulge loops are efficiently cleaved by low-molecular-weight catalysts of different nature and, particularly, by diazabicyclo[2.2.2]octane-based catalysts. Moreover, RNA can efficiently be cleaved with sufficiently high selectivity at the chosen region, in other words, site-selective RNA cleavage can be achieved by changing the RNA structure (construction of an artificial bulge loop, the use of magnesium ions).

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