Biochemistry (Moscow), Vol. 66, No. 3, 2001, pp. 345-349. Translated from Biokhimiya, Vol. 66, No. 3, 2001, pp. 425-430.

SHORT COMMUNICATIONS

A Phosphodiesterase from Ascites Carcinoma Krebs II Cells Specifically Cleaves the Bond between VPg and RNA of Encephalomyocarditis Virus

A. Yu. Gulevich, R. A. Yusupova, and Yu. F. Drygin*

Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, 119899 Russia; fax: (095) 939-3181; E-mail: drygin@genebee.msu.su

Received October 30, 2000
Revision received November 23, 2000

Abstract—The substrate specificity of the "unlinking" enzyme from ascites carcinoma Krebs II cells has been investigated. The enzyme specifically splits the interpolymeric phosphodiester bond between Kp and the 5′-terminal phosphate group of the uridylic acid residue in the Kp–pUpUpGp complex.

Key words: picornavirus, VPg–RNA, "unlinking" enzyme, substrate specificity, phosphodiesterase

The RNA of picornaviruses is linked by a phosphodiester bond to a small protein (VPg) [1-4]. VPg is associated with the 5′-end of the replicating RNA molecules [5, 6], in contrast to RNA isolated from polysomes, which lack VPg [7, 8]. Enzymatic activity catalyzing the hydrolysis of the heteropolymeric bond between VPg and RNA of the poliomyelitis virus was first discovered in HeLa cells. The cleavage of VPg from the full-size RNA was not accompanied by significant degradation of the RNA [9]. The enzymatic activity was shown both in infected and in uninfected cells. The "unlinking" enzyme was later found in mouse L cells, wheat germ, rabbit reticulocytes [10], and ascites carcinoma Krebs II cells [11]. The enzyme from HeLa cells catalyzed the unlinking of the residual K-peptide(s) associated with the poliovirus RNA after treatment of the VPg–RNA complex with proteinase K and of the full-size VPg with the same efficiency. However, the rate of VPg unlinking from the bound 5′-terminal nonanucleotide in poliovirus was 2.5 times lower than that of the original VPg–RNA, while such complexes as VPg–pU and VPg(Kp)–pUp were completely resistant to the enzyme [10]. On the other hand, in comoviruses a similar VPg–RNA complex formed via a phosphodiester bond between serine of the protein and the 5′-terminal uridylic acid of RNA was not dissociated in extracts of rabbit reticulocytes [12] and did not serve as a substrate for the "unlinking" enzyme from mouse ascites carcinoma [13].

These data indicate that, for effective cleavage of the covalent linkage unit Tyr-(5′P→O)-pU of the substrate, an extended nucleic acid region adjacent to VPg and probably only one tyrosine residue in VPg are necessary. We suggested that by gradual shortening of the 5′-terminal fragment of the picornaviral RNA, the minimum length of the nucleic component required for substrate recognition by the "unlinking" enzyme might be determined. Actually, we have recently shown for the encephalomyocarditis virus that the rate of hydrolysis of Kp–RNA and Kp–pUpUpGp by the "unlinking" enzyme is similar (unpublished data). In this paper, using a low-molecular-weight substrate, we provide direct evidence that the "unlinking" enzyme from mouse carcinoma cells selectively hydrolyzes the interpolymeric phosphodiester bond between the K-peptide and 5′-terminal trinucleotide of the RNA of the encephalomyocarditis virus and does not cleave internucleotide bonds.

Abbreviations: EMCV) encephalomyocarditis virus; VPg) viral protein genome-linked; HSIHPP) N-(4-hydroxyphenyl)propionic acid-N-hydroxysuccinimide ester; Kp) residual peptide(s) covalently bound to RNA and obtained by treatment of VPg in the VPg–RNA complex with proteinase K; PEI) polyethylene imine; HPLC) high-performance liquid chromatography.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

The following reagents and enzymes were used in this work: RNase T1, RNase A, uridine-2′,3′-cyclophosphate, and 3′-CMP were from Sigma (USA); proteinase K was from Boehringer Mannheim (Germany); polynu-

0006-2979/01/6603-0345$25.00 ©2001 MAIK "Nauka/Interperiodica"

cleotide kinase from phage T4 and calf intestinal alkaline phosphatase were from SibEnzyme (Russia); acrylamide and HSIHPP were from Serva (Germany); N,N′-methylene-bis-acrylamide was from Fluka (Switzerland); chloramine T, Kieselgel 60, and PEI-cellulose were from Merck (Germany); pUp was from P-L Biochemicals (USA); Na[^{125}I] was from Izotop (Russia); [γ-^{32}P]ATP (5,000 Ci/mmol was from Radioisotope (Russia), and Hyperfilm-MP X-ray film was from Amersham (UK). All other chemicals were of analytical grade (Reakhim, Russia).

Isolation of EMCV VPg–RNA and preparation of Kp–RNA were performed as described [11]. VPg–RNA (100 μg) was incubated with proteinase K (2 mg/ml) in buffer containing 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.5% SDS for 2 h at 37°C. After incubation, Kp–RNA was deproteinized in phenol–chloroform (9 : 1 v/v) and precipitated with ethanol. To prepare substrate, the Kp–RNA complex was labeled *in vitro* [14] using a modification of the Bolton–Hunter procedure. The labeling was carried out in two stages: the amino groups of the K-peptide(s) were first acylated with an excess of HSIHPP and then iodinated with Na[^{125}I] in the presence of chloramine T.

The "unlinking" enzyme was isolated according to a recently developed procedure [15] that includes fractionation with ammonium sulfate, pH-dependent fractionation, cation-exchange chromatography on CM-cellulose, chromatofocusing, and size-exclusion HPLC. To assay the unlinking enzyme activity, EMCV [^{125}I]Kp–RNA was used as a substrate; the products of the reaction were separated by TLC on Kieselgel in butanol-1–water–acetic acid (3.75 : 1 : 1) [13].

The complex of K-peptide(s) with the 5′-terminal trinucleotide of EMCV RNA was obtained by treatment of Kp–RNA with RNase T1. For this purpose, 20 μl of labeled [^{125}I]Kp–RNA (10 μg, ~1,650,000 cpm) and unlabeled [^{127}I]Kp–RNA complex (50 μg) in water were mixed with 5 μl of RNase T1 (1.25 units). Hydrolysis proceeded for 3 h at room temperature (20-24°C).

The hydrolysis products were separated in a 15% polyacrylamide gel containing 7 M urea. After electrophoresis, the gel was exposed to X-ray film for 40 min at 4°C. The region corresponding to [^{125}I]Kp–pUpUpGp was excised, and the substance was eluted with 125 μl of water overnight with intensive shaking at 4°C. After centrifugation at 14,000 rpm for 20 min, [^{125}I]Kp–pUpUpGp was precipitated from the supernatant by the addition of 600 μl of 2 M $LiClO_4$ and 600 μl of acetone at room temperature for 20 min. The precipitate was collected by centrifugation and stored under acetone at –20°C. Three picomoles of [^{125}I]Kp–pUpUpGp was obtained.

The hydrolysis of [^{125}I]Kp–pUpUpGp by the "unlinking" enzyme was conducted as follows: to 1 pmole of the compound in 5 μl of 0.15% aqueous Triton X-100, 40 μl of a highly purified enzyme preparation (0.02 mg/ml) in TMM/200 buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 200 mM KCl) were added, and the mixture was incubated for 1.5 h at 37°C. After hydrolysis, the "unlinking" enzyme was inactivated by heating of the reaction mixture at 56°C for 20 min. The completeness of hydrolysis of the interpolymeric phosphodiester bond was monitored by TLC of the hydrolyzate aliquots (2 μl) on Kieselgel. To dephosphorylate the trinucleotide formed (pUpUpGp), the reaction mixture (40 μl) was concentrated to 6 μl on a SpeedVac concentrator (Savant Instruments Inc., USA). One microliter of 1 M Tris-HCl buffer, pH 9.0, 1 μl of water, and 1 μl of alkaline phosphatase from calf intestine (0.215 unit) were added to the concentrated solution and incubated at 37°C for 30 min. One microliter of phosphatase solution (0.215 unit) was again added to the reaction mixture, and incubation was continued for 30 min at 37°C. The reaction was terminated by the addition of 3 μl of 1 M Tris-HCl buffer, pH 7.5, and heating at 75°C for 30 min. In the control experiment, [^{125}I]Kp–pUpUpGp was treated as described above except that the hydrolysis by the "unlinking" enzyme was omitted.

To introduce the radioactive label at the 5′-ends of the hydrolysis products, 1 μl of polynucleotide kinase T4 (4 units) in 1.5 μl of a tenfold polynucleotide kinase T4 buffer and 1 μl of [γ-^{32}P]ATP (10 μCi, 2 pmoles) were added to 11.5 μl of the resulting mixture. The reaction was allowed to proceed at 37°C for 2 h and stopped by heating at 95°C for 1 min. The treatment of [^{125}I]Kp–pUpUpG in the control experiment was conducted in a similar manner. The products of the reaction were analyzed by ascending TLC on PEI-cellulose in 1.5 M LiCl. The chromatograms were exposed to Hyperfilm-MP at room temperature for 40 min.

To digest the labeled products obtained at the previous stage by RNase A, 2 μl of the enzyme solution at a concentration of 300 μg/ml were added to the reaction mixture (13 μl) and incubated at 37°C for 30 min. The reaction products were analyzed by one-dimensional TLC on PEI-cellulose in 1.5 M LiCl. Two-dimensional separation was performed on the same sorbent as descibed below.

5′-[^{32}P]pCp was obtained by phosphorylation of 3′-CMP (500 pmoles) using [γ-^{32}P]ATP (10 μCi, 2 pmoles) in the presence of polynucleotide kinase from phage T4 (4 units) as described [16]. To characterize the resulting 5′-[^{32}P]pCp, aliquots of the compound were subjected to chromatography on PEI-cellulose in 1.5 M LiCl [17].

The mixture of 5′-[^{32}P]phosphouridine-2′,3′-cyclophosphate, 5′-[^{32}P]uridine-5′,3′-diphosphate, and 5′-[^{32}P]uridine-5′,2′-diphosphate was produced as follows: 3 μl of water and 1 μl of RNase A to a final concentration of 50 μg/ml were added to 2 μl of uridine-2′,3′-cyclophosphate (0.6 μmole). Hydrolysis was performed for 30 min at 37°C. The resulting solution containing uridine-2′,3′-cyclophosphate, uridine-3′-phosphate, and

uridine-2′-phosphate was diluted with water to a final concentration of nucleotides of 500 μM. The uridine phosphates were phosphorylated with [γ-^{32}P]ATP in the presence of polynucleotide kinase of phage T4 [16]. To the substrate solution (500 pmoles in 1 μl), 7 μl of water, 1 μl of a tenfold polynucleotide kinase buffer, 1 μl of [γ-^{32}P]ATP (10 μCi, 2 pmoles), and 1 μl of the enzyme solution (4 units) were added. The reaction proceeded for 1 h at 37°C and terminated by heating at 95°C for 1 min. To characterize the resulting 5′-[^{32}P]pUp, aliquots of the reaction mixture were subjected to chromatography on PEI-cellulose in 1.5 M LiCl. [γ-^{32}P]ATP, uridine-2′,3′-cyclophosphate, and pUp were used as markers.

Two-dimensional separation of nucleotides was performed as described [17] except that sodium formate was replaced by sodium acetate. In the first dimension, chromatography was carried out in a step LiCl gradient (2 min in 0.2 M LiCl, 6 min in 1 M LiCl, and then in 1.6 M LiCl until the front of the solvent reached 13 cm from the origin of the chromatogram). The chromatograms were air-dried; the front zone was cut off and discarded. The support was then desalted by three successive washes in ethanol and dried. In the second dimension, chromatography in sodium acetate buffer (pH 3.4) of different concentrations (0.5 M for 30 sec, 2.0 M for 2 min, and 4 M until the front of the solvent reached the distance of 15 cm from the origin of the chromatogram) was used. After the separation, the plates were dried; the position of markers was determined under short-wavelength UV-light; then the chromatograms were exposed to X-ray film.

RESULTS AND DISCUSSION

The objective of this work was to investigate the specificity of hydrolysis of the interpolymeric phosphodiester bond Tyr-(5′P→O)-pU in the substrate by the "unlinking" enzyme from ascite carcinoma Krebs II cells.

Among the products of hydrolysis of the complex between VPg and the 5′-terminal nonanucleotide of the poliovirus catalyzed by the "unlinking" enzyme from HeLa cells and purified only 250 times, a nonanucleotide was revealed [10]. Independently we showed that, after treatment of [^{3}H-Tyr]VPg–RNA complex with the "unlinking" enzyme followed by proteinase K, most K-peptides were not linked to the nucleotides.

To determine the specificity of the "unlinking" enzyme in the isolated preparation, it was necessary to avoid contamination with endo- and exonucleases. We showed that the "unlinking" enzyme from the ascite carcinoma cells was contaminated with a purine-specific nuclease (Gulevich et al., unpublished data). However, we also demonstrated that Kp–pUpUpGp, the product of complete hydrolysis of EMCV Kp–RNA by RNase T1, as well as other high-molecular-weight products were good substrates for the "unlinking" enzyme. Thus, the contaminating purine-specific endonuclease could not interfere with the analysis of hydrolysis of the interpolymeric phosphodiester bond in the covalent linkage unit by the "unlinking" enzyme.

To independently confirm specific hydrolysis of the phosphodiester bond in the covalent linkage unit seen earlier using high-molecular-weight substrates selectively labeled in the peptide part [13, 14], in this work we identified a nucleotide directly linked with the peptides using a relatively simple substrate.

For this purpose, EMCV [^{125}I]Kp–RNA was treated with RNase T1 in the presence of a fivefold excess of [^{127}I]Kp–RNA (identical substrate but iodinated with unlabeled iodine, which was added for kinetic reasons to obtain greater amounts of the desired product) under conditions providing complete hydrolysis; as a result, the Kp–pUpUpGp complex was obtained. The complex between the K-peptide(s) and the trinucleotide was isolated by electrophoresis of the reaction products in a 15% polyacrylamide gel containing 7 M urea. The compound was eluted from the corresponding zone of the gel (Fig. 1) and precipitated with $LiClO_4$ in acetone.

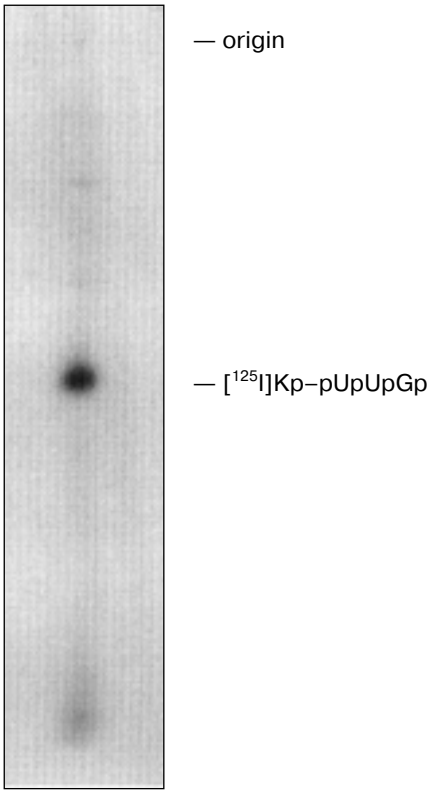

Fig. 1. Electrophoretic separation of the products of complete hydrolysis of EMCV [^{125}I]Kp–RNA by RNase T1 in 15% polyacrylamide gel in the presence of 7 M urea.

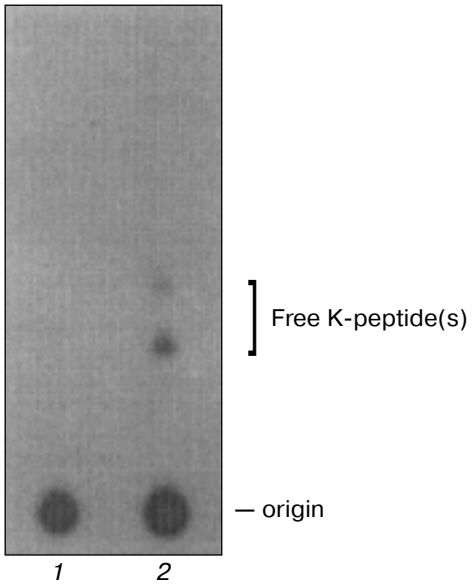

Fig. 2. TLC on Kieselgel of the products of [^{125}I]Kp–pUpUpGp hydrolysis by the "unlinking" enzyme: *1*) control after incubation in the reaction buffer; *2*) after treatment with the "unlinking" enzyme.

The reaction product was hydrolyzed by the "unlinking" enzyme (Fig. 2). The yield of free K-peptide(s) and the respective trinucleotides was ~20%. The low recovery was due to the conditions of the hydrolysis.

As seen from Fig. 2, no intermediate products of nonspecific nuclease hydrolysis of the heterocomplex (Kp–pUp, Kp–pU, and Kp–p), which had much lower chromatographic mobilities in the system used [18], were formed by the treatment with the enzyme.

Since the enzyme was sensitive to temperature [11], we inactivated it by heating at 56°C after the reaction. The resulting mixture consisting of free K-peptide(s), an expected trinucleotide pUpUpGp, and a nonhydrolyzed heterocomplex was concentrated, dephosphorylated by alkaline phosphatase from calf intestine, and then phosphorylated with [γ-^{32}P]ATP in the presence of polynucleotide kinase (see "Materials and Methods").

It is noteworthy that the latter treatment, except for [γ-^{32}P]ATP derivatives, resulted in the formation of a single labeled product, 5′-[^{32}P]pUpUpG. This finding proved the absence of endo- and exonucleases hydrolyzing the 5′-terminal trinucleotide in the enzyme preparation. The phosphatase treatment of the mixture containing free K-peptides, trinucleotides, and the original Kp–pUpUpGp complex produced only one substrate for polynucleotide kinase, UpUpG. If the products of complete hydrolysis of the trinucleotide (pU, pUp, Up, pG, pGp, and Gp) were present in the mixture, the treatment with phosphatase would lead to the formation of the corresponding nucleosides, which are not phosphorylated by polynucleotide kinase. On the other hand, if nonspecific hydrolysis occurred, UpU or UpG could be formed after dephosphorylation and serve as acceptors for phosphorylation by polynucleotide kinase. In this instance, Kp–p, Kp–pU, and/or Kp–pUp would be detected among the products of Kp–pUpUpGp hydrolysis. However, as shown earlier, these compounds were not found (Fig. 2). Consequently, dinucleoside phosphates (UpU and UpG) capable of participating in the phosphorylation reaction were not present in the reaction mixture.

Thus, the formation of labeled 5′-[^{32}P]pUpUpG could result only from the specific cleavage of the bond between the K-peptide and pUpUpGp catalyzed by the "unlinking" enzyme. Further evidence in favor of this suggestion was obtained by digestion of 5′-[^{32}P]pUpUpG with RNase A. The formation of three labeled products, 5′-[^{32}P]pU-3′-phosphate, 5′-[^{32}P]pU-2′-phosphate, and 5′-[^{32}P]pU-2′,3′-cyclophosphate, was expected. One-dimensional separation of the reaction products on PEI-cellulose showed that the compounds formed from the 5′-terminal trinucleotide and the markers produced independently by labeling uridine-2′,3′-cyclophosphate treat-

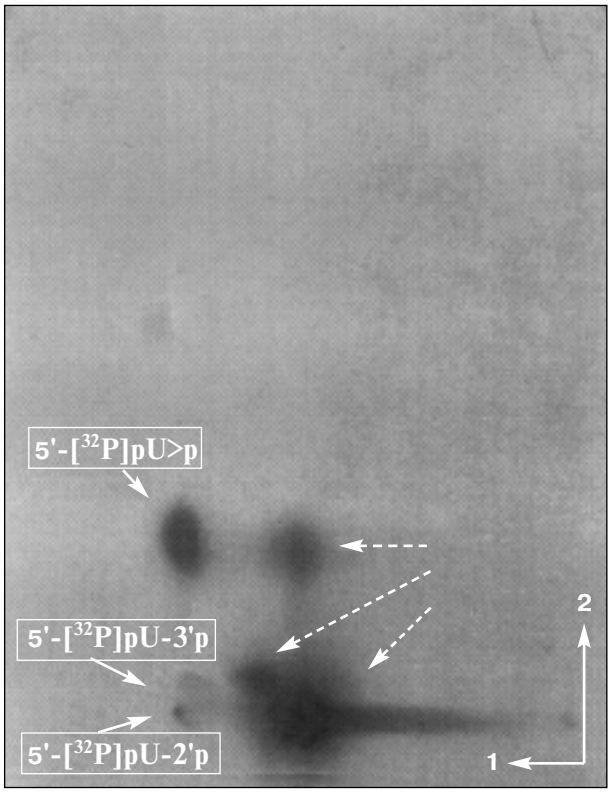

Fig. 3. Two-dimensional chromatography on PEI-cellulose of 5′-[^{32}P]pUpUpG hydrolyzed by RNase A. Solid arrows indicate the positions of 5′-[^{32}P]pU-3′-phosphate, 5′-[^{32}P]pU-2′-phosphate, and 5′-[^{32}P]pU-2′,3′-cyclophosphate; broken arrows show the products of [γ-^{32}P]ATP conversion. The zone corresponding to 5′-[^{32}P]pU-3′-phosphate coincides with the unlabeled marker.

ed with RNase A with 5′-[^{32}P]phosphate had identical mobility. However, it should be noted that the mobility of 5′-[^{32}P]pUp and 5′-[^{32}P]pUpUpG (with similar charges but different masses) during one-dimensional chromatography in 1.5 M LiCl is similar.

By high-resolution two-dimensional TLC of the reaction products on PEI-cellulose, we showed the predominant formation of 5′-[^{32}P]pU-2′,3′-cyclophosphate and of small amounts of 5′-[^{32}P]pU-3′- and 5′-[^{32}P]pU-2′-phosphates (Fig. 3).

The predominant formation of cyclophosphate can be explained by high concentrations of potassium and magnesium salts (~600 mM KCl) in the reaction mixture. It is well known that hydrolysis of internucleotide bonds in pyrimidine-containing polynucleotides by RNase A proceeds through the intermediate formation of 2′,3′-pyrimidine cyclophosphates [19]. The optimum concentration of univalent metal salts for RNase A is about 30 mM [20], while higher salt concentrations in the reaction mixture decrease the rate of the catalytic reaction [21]. Furthermore, divalent metal ions at a concentration of $5 \cdot 10^{-4}$ M also reduce the RNase A activity [21].

The results indicate that the formation of labeled 5′-[^{32}P]pUp after the above-mentioned treatments could occur only by specific excision of a free K-peptide from [^{125}I]Kp–pUpUpGp catalyzed by the "unlinking" enzyme and accompanied by the formation of a trinucleotide, which became the substrate for polynucleotide kinase after the treatment with phosphatase. The formation of labeled 5′-[^{32}P]pUpU, the product of partial nonspecific hydrolysis of [^{125}I]Kp–pUpUpG(p), was not observed. Additionally, the kinetics of VPg unlinking studied earlier showed that only a purine-specific nuclease was present in the "unlinking" enzyme preparation (unpublished data). Thus, the "unlinking" enzyme from Krebs II cells selectively hydrolyzes the phosphodiester bond in the [^{125}I]Kp–pUpUpG(p) complex and does not affect the internucleotide bonds.

The whole body of evidence indicates that the "unlinking" enzyme belongs to a new type of phosphodiesterases. The targets for the enzyme in the cell are unknown. In the virology of picornaviruses, it has been speculated that the "unlinking" enzyme is involved in regulation of replication, translation, and capsidation of viral RNA [10]. Alternatively, the "unlinking" enzyme may resemble the cellular mRNA-decapping enzyme and trigger decay of the virus message in a similar manner. As well documented, the decapping enzyme triggers 5′→3′ exonuclease degradation of the cellular mRNAs. The decapping enzyme exhibits unique specificity with respect to a pyrophosphate bond in the cap structure and does not hydrolyze internucleotide bonds [22]. In addition, the decapping enzyme preferably reacts with extended nucleic substrates associated with cap structures [23].

The authors are grateful to the staff of the Department of Radio Isotope Analysis of the Belozersky Institute of Physico-Chemical Biology for assistance and to E. A. Kubareva for the preparations of polynucleotide kinase and alkaline phosphatase.

This work was supported in part by the Russian Foundation for Basic Reseach (grant No. 97-4-49763).

REFERENCES

1. Lee, Y. F., Nomoto, A., and Wimmer, E. (1976) *Progr. Nucl. Acid. Res. Mol. Biol.*, **19**, 89-96.
2. Flanegan, J. B., Petterson, R. F., Ambros, V., Hewlet, M. G., and Baltimore, D. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 961-965.
3. Sangar, D. V., Rowlands, D. L., Harris, T. J. R., and Brown, F. (1977) *Nature*, **268**, 648-650.
4. Drygin, Yu. F., Vartapetian, A. B., and Chumakov, K. M. (1979) *Mol. Biol. (Moscow)*, **13**, 777-787.
5. Takegami, T., Semler, B. L., Anderson, C. W., and Wimmer, E. (1983) *Virology*, **128**, 33-47.
6. Vartapetian, A. B., Koonin, E. V., Agol, V. I., and Bogdanov, A. A. (1984) *EMBO J.*, **3**, 2583-2588.
7. Hewlet, M. J., Rose, J. K., and Baltimore, D. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 327-330.
8. Nomoto, A., Lee, Y. F., and Wimmer, E. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 375-380.
9. Ambros, V., Petterson, R. F., and Baltimore, D. (1978) *Cell*, **15**, 1439-1446.
10. Ambros, V., and Baltimore, D. (1980) *J. Biol. Chem.*, **255**, 6739-6744.
11. Drygin, Yu. F., and Siyanova, E. Yu. (1986) *Biokhimiya*, **51**, 249-258.
12. De Varennes, A., Lomonosoff, G. P., Shanks, M., and Maule, A. J. (1986) *J. Virol.*, **27**, 2347-2351.
13. Drygin, Yu. F., Shabanov, A., and Bogdanov, A. (1988) *FEBS Lett.*, **239**, 343-346.
14. Shabanov, A. A., Kalyuzhnyi, A. A., Gottich, M. B., and Drygin, Yu. F. (1996) *Biochemistry (Moscow)*, **61**, 1106-1118 (Russ.).
15. Yusupova, R. A., Gulevich, A. Yu., and Drygin, Yu. F. (2000) *Biochemistry (Moscow)*, **65**, 1219-1226.
16. Maniatis, T., Fritsch, E., and Sambrook, J. (1984) *Molecular Cloning* [Russian translation], Mir, Moscow.
17. Belozersky, A. N. (ed.) (1970) in *Methods of Nucleic Acid Analysis* [in Russian], Mir, Moscow, pp. 44-45.
18. Drygin, Yu. F., Shabanov, A. A., and Bogdanov, A. A. (1988) *Biokhimiya*, **53**, 1371-1379.
19. Shapot, V. S. (1968) *Nucleases* [in Russian], Meditsina, Moscow.
20. Sideris, D. S., and Fragoulis, E. G. (1987) *Eur. J. Biochem.*, **164**, 309-315.
21. Weickmann, J. L., Elson, M., and Glitz, D. G. (1981) *Biochemistry*, **20**, 1272-1278.
22. Stevens, A. (1988) *Mol. Cell. Biol.*, **8**, 2005-2010.
23. Stevens, A. (1980) *Biochem. Biophys. Res. Commun.*, **96**, 1150-1155.