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Complex of the Herpes Simplex Virus Initiator Protein UL9 with DNA as a Platform for the Design of a New Type of Antiviral Drugs

A. N. Surovaya[a], S. L. Grokhovsky[a], Ya. G. Gursky[b], V. L. Andronova[c], V. S. Arkhipova[a], N. P. Bazhulina[a], G. A. Galegov[c], and G. V. Gursky[a]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 119991 Russia*
E-mail: annasur@eimb.ru
[b] *Scientific and Technological Cardiology Complex, Moscow, 121552 Russia*
[c] *Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Moscow, 123098 Russia*

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Abstract—The protein binding to the origin of replication of the herpes simplex virus type 1 is DNA helicase encoded by the UL9 gene of the herpes virus. The protein specifically binds to two binding sites in the viral DNA replication origins OriS or OriL. In order to determine the role of the UL9 protein in the initiation of replication and find efficient inhibitors of the UL9 activity, we have synthesized a recombinant UL9 protein expressed in *E. coli* cells. It was found that the recombinant UL9 protein binds to Boxes I and II in OriS and possesses DNA helicase and ATPase activities. In the complex with a fluorescent analog of ATP, two molecules of the ATP analog bind to one protein dimer molecule. It was also found that the UL9 protein in the dimer form can bind simultaneously to two DNA fragments, each containing specific binding sites for the protein. The interaction of the recombinant UL9 protein with the 63-mer double- and single-stranded oligonucleotides OriS and OriS*, which correspond to the origin of replication of herpes simplex virus, has been investigated. From the titrations of OriS and OriS* with ethidium bromide in the presence and absence of the UL9 protein, the equilibrium affinity constants of the protein binding to OriS and OriS* have been determined. A DNase I footprinting study showed that bis-netropsins exhibit preference for binding to the AT cluster in the origin of replication OriS and inhibit the fluctuation opening of AT base pairs in the AT cluster. The drugs also prevent formation of an intermediate conformation of OriS* that involves a disordered tail at the 3' end and stable Box I—Box III hairpin to which the UL9 helicase selectively binds. The stabilization by bis-netropsins of the AT-rich hairpin at its 3' end can inhibit the helicase activity. It was concluded that the antiviral activity of bis-netropsins may be associated with the inhibitory effects of bis-netropsins on these two stages of the reaction catalyzed by helicase UL9.

Key words: helicase of herpes simplex virus, replication origin, binding constant, ATPase and helicase activities, antiviral drugs, bis-netropsins

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INTRODUCTION

One of the most significant discoveries of the recent years is the establishment of the three-dimensional structure of specific complexes of DNA with the proteins playing a key part in the processes of DNA transcription and replication, among them the complexes with proteins participating in replication initiation. Examples may be DNA helicases—proteins providing for untwisting of double-stranded DNA at the expense of the energy of ATP hydrolysis [1–7]. DNA and RNA helicases operate practically in all important biological processes taking place with participation of nucleic acids. Examples are processes of DNA replication, repair and recombination, processes of regulation of gene activity and remodeling of chromatin structure. The 3D structure has been established for some DNA helicases and their complexes with DNA, nonhydrolyzable analog of ATP and ADP [4–7]. Characteristic motifs have been revealed for helicase binding and significant similarity has been established in 3D structures of a number of helicases [5–7]. However the mechanisms of action of these proteins have been studied totally insufficiently. Of significant interest is establishment of the structure of DNA complexes with initiator proteins capable of selectively binding with the origin of replication of herpes simplex virus and SV40 and initiating the process of DNA untwisting [1–3, 8, 9]. The herpesvirus helicase UL9 binds selectively with two DNA regions in the replication origin, which are at the ends of an AT cluster containing 18 (OriS) and 20 (OriL) AT basepairs. The protein possesses ATPase and helicase

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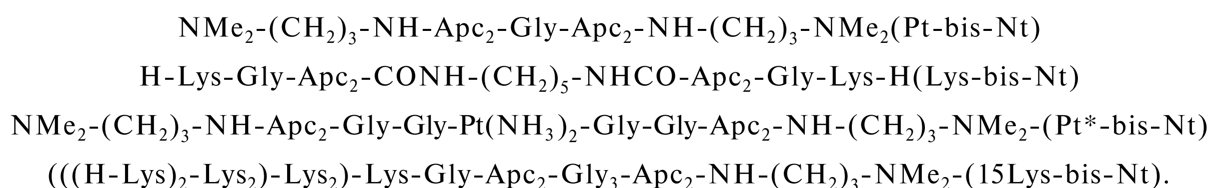
activities [1–3]. In the presence of ATP and another viral protein ICP8 (single-stranded DNA binding protein) the initiator protein UL9 causes untwisting of DNA in OriS and OriL, which leads to initiation of replication of viral DNA [1–3, 10–14]. In this work, in order to investigate the role in replication initiation of protein UL9 and to conduct a search for inhibitors of its activity, we obtained a recombinant form of protein UL9 synthesized in *E. coli* cells. We found that the full-sized recombinant protein UL9 (L-UL9) and its shortened variant S-UL9 containing 317 C-terminal amino acid residues of protein UL9 bind selectively with sites I and II in OriS. The full-sized recombinant protein L-UL9 possesses also helicase and ATPase activities.

The medicinal drugs currently used for treating infections caused by herpesvirus (acyclovir and its analogs) do not relieve patients of the relapsing character of the course of disease and appearance of virus variants resistant to acyclovir and its analogs. One of the possible ways of affecting the viral infection is the use of compounds selectively binding to viral DNA and inhibiting the activity of herpesvirus UL9 helicase. We have earlier found that dimeric derivatives of netropsin selectively bind to the A+T cluster in the herpesvirus replication origin and effectively suppress the reproduction of herpes simplex virus type I in cultured Vero cells and in experiments on laboratory animals [15–18]. Search for compounds the action of which is targeted on the specific complex of UL9 helicase with the herpesvirus replication origin presents significant interest for molecular biology and medicine.

EXPERIMENTAL

Ligands. chemical structures of netropsin (Nt) and bisNt used in the present work are presented below:



NMe_2-$(CH_2)_3$-NH-Apc_2-Gly-Apc_2-NH-$(CH_2)_3$-NMe_2(Pt-bis-Nt)

H-Lys-Gly-Apc_2-CONH-$(CH_2)_5$-NHCO-Apc_2-Gly-Lys-H(Lys-bis-Nt)

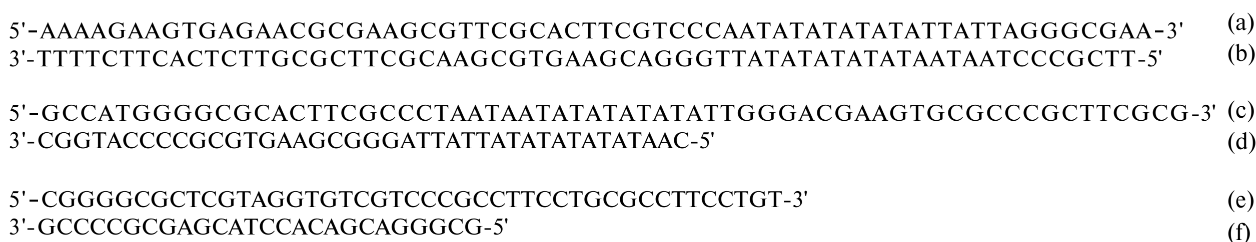
NMe_2-$(CH_2)_3$-NH-Apc_2-Gly-Gly-Pt$(NH_3)_2$-Gly-Gly-Apc_2-NH-$(CH_2)_3$-NMe_2-(Pt*-bis-Nt)

(((H-Lys)$_2$-Lys$_2$)-Lys$_2$)-Lys-Gly-Apc_2-Gly$_3$-Apc_2-NH-$(CH_2)_3$-NMe_2-(15Lys-bis-Nt).

Here Apc is the residue of 1-N-propyl-4-aminopyrrole-2-carboxylic acid, DTPA is the residue of diethylene triaminopentaaceric acid. Pt-bis-Nt and Pt*-bis-Nt contain a *cis*-diaminoplatinum group in the connecting chain between the two Nt fragments. Pt-bis-Nt and Pt*-bis-Nt were synthesized in accordance with the procedure described in work [19]. The distinction of each di-N-propylpyrrolecarboxamide fragment of the bisNt molecule from Nt consists in replacement of the C-terminal amidine group with a tertiary amine residue, replacement of the guanidylacetic acid residue present in Nt with glycine residues, and also in replacement of N-methylpyrrole cycles with N-propylpyrrole ones. These replacements increase the stability of compounds against degradation in aqueous solutions. The concentrations of bisNt were determined spectrophotometrically, with the use of a molar extinction coefficient at 297 nm equal to 42 000 $M^{-1}cm^{-1}$.

Recombinant proteins L-UL9 and S-UL9. The full-sized recombinant protein L-UL9 and its shortened variant S-UL9 containing the C-terminal DNA-binding domain of the L-UL9 protein were purified with the aid of a metal-chelating (Ni-NTA) column (Quiagen). Protein concentrations were determined spectrophotometrically, using the values of molar extinction coefficients at 280 nm equal to 89 000 and 35 400 M^{-1} cm^{-1} for monomers of L-UL9 and S-UL9 respectively. Proteins L-UL9 and S-UL9 were stored in buffer A containing 20 mM Tris-HCl (pH 7.2); 20 mM HEPES–NaOH; 0.54 M NaCl; 0.01% Tween 20; 0.10 mM EDTA; 1 mM dithiothreitol and 20% (vol/%) glycerol.

Oligonucleotides. In the capacity of substrate for investigating the DNA-binding and DNA-untwisting activities of the recombinant L-UL9 protein we used the following oligonucleotide constructs:



5'-AAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAA-3' (a)
3'-TTTTCTTCACTCTTGCGCTTCGCAAGCGTGAAGCAGGGTTATATATATATAATAATCCCGCTT-5' (b)

5'-GCCATGGGGCGCACTTCGCCCTAATAATATATATATATTGGGACGAAGTGCGCCCGCTTCGCG-3' (c)
3'-CGGTACCCCGCGTGAAGCGGGATTATTATATATATATAAC-5' (d)

5'-CGGGGCGCTCGTAGGTGTCGTCCCGCCTTCCTGCGCCTTCCTGT-3' (e)
3'-GCCCCGCGAGCATCCACAGCAGGGCG-5' (f)

oligonucleotides (a–f) were synthesized by the phosphoramidate method and purified with the aid of electrophoresis in polyacrylamide gel (Sintol, Russia). For formation of a 63-membered duplex consisting of oligonucleotides (a) and (b), complementary strands were mixed at equimolar concentrations, mixtures were annealed at 90°C, and then cooled to room temperature over 8–10 h. The nucleotide sequence of the 63-membered duplex corresponds to the fragment of DNA in the replication origin OriS of herpes simplex virus type I. This fragment includes the A + T cluster and binding sites I and III for the initiator protein UL9. For formation of oligonucleotide construct containing oligonucleotides (c) and (d) or (e) and (f), equimolar mixtures of corresponding oligonucleotides were annealed at 90°C, and then cooled over 8–10 h to room temperature. The double-stranded part of the oligonucleotide construct consisting of oligonucleotides (c) and (d) contains the A + T cluster and the specific binding site II for the initiator protein UL9. It also has an extended single-stranded "tail" at the 3' end of oligonucleotide (c). As regards the oligonucleotide construct formed by oligonucleotides (e) and (f), it does not contain specific binding sites for protein UL9. This construct has earlier been used by Lee and Lehman for investigating the helicase activity of protein UL9 in the absence of viral protein ICP8 [10]. In solution at room temperature the single-stranded oligonucleotide (a) spontaneously forms two hairpins: the GC-rich hairpin that is stabilized at the expense of formation of complementary basepairs between nucleotides present in boxes I and III, and the AT-rich hairpin stabilized at the expense of formation of complementary pairs between bases of the A + T cluster and the GC basepairs flanking it at both sides. Oligonucleotide (a) was annealed in 0.001 M cacodylate buffer (pH 7.0) in the presence of 0.1 M LiCl. After annealing at 97°C for five minutes the solution was immediately placed into ice in order to increase the probability of formation of hairpin structures and kept at 0°C for 10 min.

DNase footprinting. The KpnI–PstI fragment of viral DNA (≈170 bp) containing OriS was cloned in a modified plasmid PGEM7z(f+) [20]. The longer PvuII–HindIII fragment of this plasmid containing a viral DNA fragment was used to study the binding of proteins L-UL9 and S-UL9 to OriS with the aid of the DNase footprinting method [21]. To prepare complexes with proteins L-UL9 and S-UL9 10 μL of a solution of DNA fragment containing a radioactive label at the end (gamma ^{32}P, approximately 10^4 Bq) with a concentration of ~20 μM(bp) in 10 mM Tris-HCl (pH 7.0), 0.10 M NaCl and 10 μg/mL BSA was mixed with 10 μL of L-UL9 or S-UL9 solutions in 20 mM Tris-HCl buffer pH 7.2, 0.01% Tween 40, 10% (vol/%) glycerol, 1 mM β-mercaptoethanol, 12 mM $MgCl_2$ and 0.10 mM ATP and kept at 0°C for 12 h. The mixture was supplemented with 20 μL of DNase I solution (≈0.5 μg/mL) in 20 mM Tris-HCl (pH 7.2), 0.10 M NaCl, 5 mM $MnCl_2$ and kept for 3 min at 0 C. To stop the reaction the mixture was supplemented with 60 μL of a solution of 0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 10 μg/mL tRNA. The mixture was extracted with phenol, DNA was precipitated with ethanol, washed with 70% ethanol, dried, dissolved in 1 μL 95% formamide containing 15 mM EDTA (pH 8.0), 0,05% Bromphenol Blue and 0.05% xylene cyanole FF, heated for q min at 90°C, rapidly cooled to 0°C and applied onto denaturing polyacrylamide gel 40 cm long with gradient thickness 0.15–0.45 mm. Electrophoresis was conducted for 50 min at 100 W (2.5 kV) at a temperature of 60–70°C. Before exposure the gel was fixed in 10% acetic acid and dried on glass pretreated with ?-methacrylpropyloxysilane (LKB, Sweden) and exposed with a luminescent screen with subsequent scanning on a Cyclone Storage Phosphor System (Packard BioScience Company, USA).

Helicase activity. In experiments on DNA untwisting by the initiator protein UL9 as substrates we used oligonucleotide constructs (a)+(b), (c)+(d) and (e)+(f). At a temperature of 42°C on average three basepairs in duplex (a)+(b) are present in untwisted state and can serve as a "nucleus" for the helicase-catalyzed "helix–coil" transition in the A+T cluster. In the capacity of a substrate for helicase L-UL9 we also used oligonucleotide constructs consisting of oligonucleotides (c) and (d), and also (e) and (f). Each of these constructs has a single-stranded tail at the 3' end of the upper strand. Transfer of the radioactive phosphate group from gamma32P-ATP onto the 5' end of oligonucleotides was performed with the help of T4 polynucleotide kinase (Sibenzim, Russia) as described in [20]. After purification each of the double-stranded labeled oligonucleotides (a)+(b), (c)+(d) and (e)+(f) were fused in equimolar amounts in buffer containing 20 mM Tris-HCl (pH 8.0) in the presence of 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.01% Triton X-100, 10% (vol/%) glycerol, 0.27 mM ATP and supplemented with 1 μL helicase L-UL9 (7.5 μM) in 100 mM Tris-HCl buffer (pH 7.8) in the presence of 5 mM dithiothreitol, 20 mM $MgCl_2$, 50% (vol/%) glycerol, 50 μg/mL calf serum albumin, 0.05% Triton X-100 and 0.27 mM ATP. Reaction mixtures were kept for 60 min at 42°C. Samples were applied onto nondenaturing 10% polyacrylamide gel (0.4 mm × 45 cm). Electrophoresis was conducted for 2 h 10 min at 650 V, 10–15 W, gel temperature ~22°C.

Equipment and methods. Circular dichroism spectra were measured with a Jasco-720 instrument, using cells with optical path length of 1.0, 0.1 and 0.2 cm. Absorption spectra were measured with a Jasco V-550 spectrophotometer. Fluorescent measurements were conducted with a Cary Eclipse spectrofluorimeter. Investigations were conducted in 0.001 M Na-cacodylate buffer in the presence of 0.1 M NaCl, pH 7.0. Bis-Nts were dissolved in a small amount of

ethanol and than transferred into the above-mentioned buffer.

RESULTS AND DISCUSSION

Obtaining recombinant protein L-UL9 and its shortened variant S-UL9. In order to explore what role in replication initiation is played by protein UL9 and to conduct a search for inhibitors of its activity, we cloned the gene of UL9 of herpes simplex virus type I and obtained a recombinant form of the UL9 protein synthesized in *E. coli* cells. The fragment of viral DNA (strain L_2 from the Collection of the Ivanovsky Institute of Virology, RAMS) containing the UL9 gene was amplified with the aid of PCR in the form of three overlapping fragments with the use of the following primers:

5'-CGGATCCGCATATGCCTTTCGTGGGGGGCGCGGAGT-3' (1)

5'-AGCAACAGCACGCGGTCCGTAAACTGA-3' (2)

5'-CGTGGGCCCCAACCTTCTGAACAACTA-3' (3)

5'-GACATCGGACCGGAGGTATTTTCGAC-3' (4)

5'-CTGTTTTTGCGGGGCGTACATTTCGAC-3' (5)

5'-GTTGGTACCTTATAGGGTGCTAAAGTTCACCGCCCCCTGCATCATGG-3' (6)

Each of the amplified fragments was cloned and sequenced. Since the UL9 gene has a high content of GC basepairs, PCR was conducted with the use of Pfu DNA polymerase in the presence of Thermus Aq. SSB protein (single-stranded DNA binding protein). After assembly of the gene and determination of the complete nucleotide sequence the viral DNA fragment was incorporated between NdeI and KpnI sites into plasmid pET14 (Novagen) (Fig. 1).

Between the NcoI and NdeI sites an oligonucleotide was also incorporated encoding a fragment of six histidine residues. The nucleotide sequence close to the initiation codon was as follows:

M G S S H H H H H H S S G L V P R G S H M P F V G G A
5'-ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCCTTTCGTGGGGGGCGC-3'

Underlined is the sequence in the region starting from which the sequence corresponds to the UL9 gene of herpes simplex virus (strain L_2). Also indicated is the amino acid sequence at the N end of the recombinant protein.

To obtain the protein variant containing the C-terminal DNA-binding domain (corresponding to the BamHI–KpnI fragment of viral DNA), we replaced the NdeI–BamHI fragment with a short adapter oligonucleotide with retention of the sequence near the initiation codon (Fig. 2).

The nucleotide sequence near the initiation codon:

M G S S H H H H H H S S G L V P A G S H M L E D P E
5'-ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCTCGAGGATCCCGAG-3'

As previously, underlined is the sequence in the region starting from which the nucleotide sequence corresponds to the UL9 protein gene of herpes simplex virus. Upon comparison of the nucleotide sequences of the UL9 gene of the variant of herpes simplex virus L_2 from the Collection of the Institute of Virology, RAMS and the UL9 gene of the standard strain of herpes simplex virus type I (GenBank NC_001806) we disclosed several nucleotide substitutions. The majority of these substitutions do not lead to a change of the protein amino acid sequence because they are encountered in the third position of the anticodon. However three nucleotide substitutions lead to changes of the amino acid residues: Glu280Asp, Leu531Pro and Ala797Val. These changes were observed in four clones upon three independent PCR reactions, which is evidence of natural polymorphism. The L-UL9 and S-UL9 genes were expressed under control of a promoter for T7 RNA polymerase in the cells of *E. coli* (strain BL21 (DE3)) cotransformed with a helper plasmid Kan^R p15A, which is a derivative of plasmid pACYC184 (Novagen)

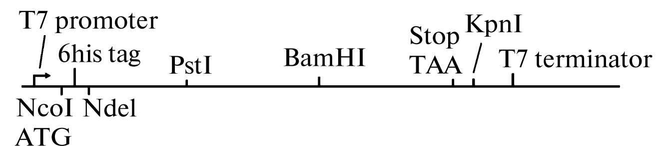


Fig. 1. Gene structure for recombinant full-sized protein UL9 (L-UL9).

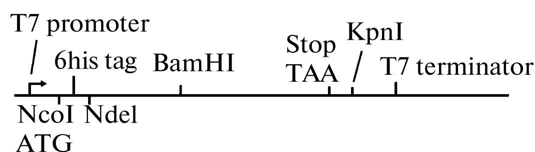


Fig. 2. Gene structure for shortened recombinant protein UL9 (S-UL9) containing the C-terminal DNA-binding domain of the UL9 protein.

and contains genes encoding the *lac* repressor and tRNA-Arg[4] of *E. coli*. Induction was conducted at 28°C by adding isopropyl-1-thio-β-D-galactopyranoside to 0.5 mM. More than 90% of protein proved to be in the water-insoluble fraction, the soluble protein was purified consecutively on a metal-chelating column (Ni-NTA) and an ion-exchange column with phosphocellulose P11. The yield of soluble protein constituted approximately 80 μg per liter of culture. The product of LUL9 gene expression is a protein of mol. mass 94 kDa. The method of obtaining a recombinant UL9 protein of herpes simplex virus in *E. coli* cells has been described by the authors of work [22]. These authors used the UL9 protein synthesized in *E. coli* cells for investigating the influence of various kinases on protein activity.

Specificity of L-UL9 and S-UL9 binding with replication origin OriS. In Fig. 3 diagrams are presented for DNase footprinting for complexes of full-sized recombinant protein (L-UL9) and its shortened variant S-UL9 with a fragment of viral DNA containing the replication origin OriS (170 bp).

As evident from Fig. 3, recombinant proteins L-UL9 and S-UL9 bind selectively with binding sites I and II in OriS. This is consistent with the data obtained earlier for binding of herpesvirus protein UL9 and its shortened fragment containing the C-terminal DNA-binding domain [1, 2, 23]. At low binding levels L-UL9 does not protect from DNase cleavage the AT cluster in OriS. Binding of L-UL9 with DNA in the presence of ATP is accompanied by conformational changes [24]. This manifests itself in that in the diagrams of footprinting for the complex of L-UL9 with OriS there appear regions with higher than control sensitivity to DNase I (Fig. 3).

Initiator protein L-UL9 binds simultaneously with two specific DNA fragments. To correlate the contributions of specific and nonspecific interaction during helicase binding with DNA we investigated the interaction of protein with specific DNA fragments (containing the replication origin OriS) and nonspecific DNA fragment, which does not contain specific binding sites for helicase. The source of specific sequences was a modified plasmid pTZ18 containing a cloned fragment of OriS of herpesvirus DNA. The product of PCR amplification of the OriS-containing insert has a size of 400 bp. This DNA region can by itself serve in the capacity of a specific DNA fragment in exploring the specific binding of helicase L-UL9 with DNA. After cleavage of this fragment with restriction endonuclease HindIII we obtained two fragments: specific fragment (270 bp) containing specific binding sites I, II and III for helicase L-UL9, and nonspecific fragment 130 bp in length. Introduction of radioactive label at the 5' ends of these fragments was conducted with the help of T4 polynucleotide kinase and gamma-^{32}P-ATP (about 2×10^4 Bq). After purification on a column with Sephadex G50 the concentration of labeled and nonlabeled DNA fragments was determined by their absorption at 260 nm. Binding of protein with preliminarily mixed specific and nonspecific labeled, or nonlabeled fragments was conducted in buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM Na-HEPES; 0.5 mM Na-EDTA, 1 mM β-mercaptoethanol, 12 mM $MgCl_2$; 100 μM ATP; 0.01% Tween 20, 10% (vol/%) glycerol, 100 μg/mL calf serum albumin. The compositions of mixtures of helicase and DNA fragments are presented in the caption to Fig. 4. After 20 min of incubation at 37°C the mixtures were introduced into wells of a 1.8% agarose gel. The agarose gel and the electrophoretic bugger contained 0.01% Tween 20; 50 mM Tris-acetate buffer; 1 mM Na-EDTA, pH 7.2. Electrophoresis was conducted at room temperature for two hours. After fixation the gel was dried and analyzed on a Phosphoimager. The results of experiments are presented in Fig. 4.

The data on the mobility of DNA fragments in agarose gel in the presence of the L-UL9 protein indicate that the L-UL9 protein can interact simultaneously with two specific DNA fragments. This manifests itself in that upon addition of nonlabeled specific DNA fragment (400 bp) containing the replication origin there is a decrease in the concentration of labeled specific duplex (270 bp), which binds with the protein simultaneously with the nonlabeled specific fragment (400 bp), while the concentration of the labeled nonspecific duplex (130 bp) changes insignificantly (Fig. 4, lanes *5* and *6*).

Untwisting of DNA oligomers by helicase L-UL9. It is known that in the presence of ATP and another viral protein ICP8 the initiator protein completely untwists the minimal duplex (80 bp) containing the replication origin and forms a strong and specific complex with the upper strand of OriS duplex, which folds into a hairpin containing an unstructured single-stranded tail at the 3' end. Untwisting of the specific DNA fragment by helicase UL9 in the absence of viral protein ICP8 is a long process, which can be investigated spectrophotometrically at 260 nm, as well as with the aid of measuring the mobility of DNA fragments in polyacrylamide gel. The authors of work [10] have found that in the absence of ICP8 the nonspecific duplex (e)+(f) is untwisted by helicase UL9, which manifests itself as the increase in optical density at 260 nm upon incubation of duplex (e)+(f) with herpesvirus helicase UL9 in the presence of 5×10^{-3} M Mg-ATP at 42°C. Under these conditions we observe an

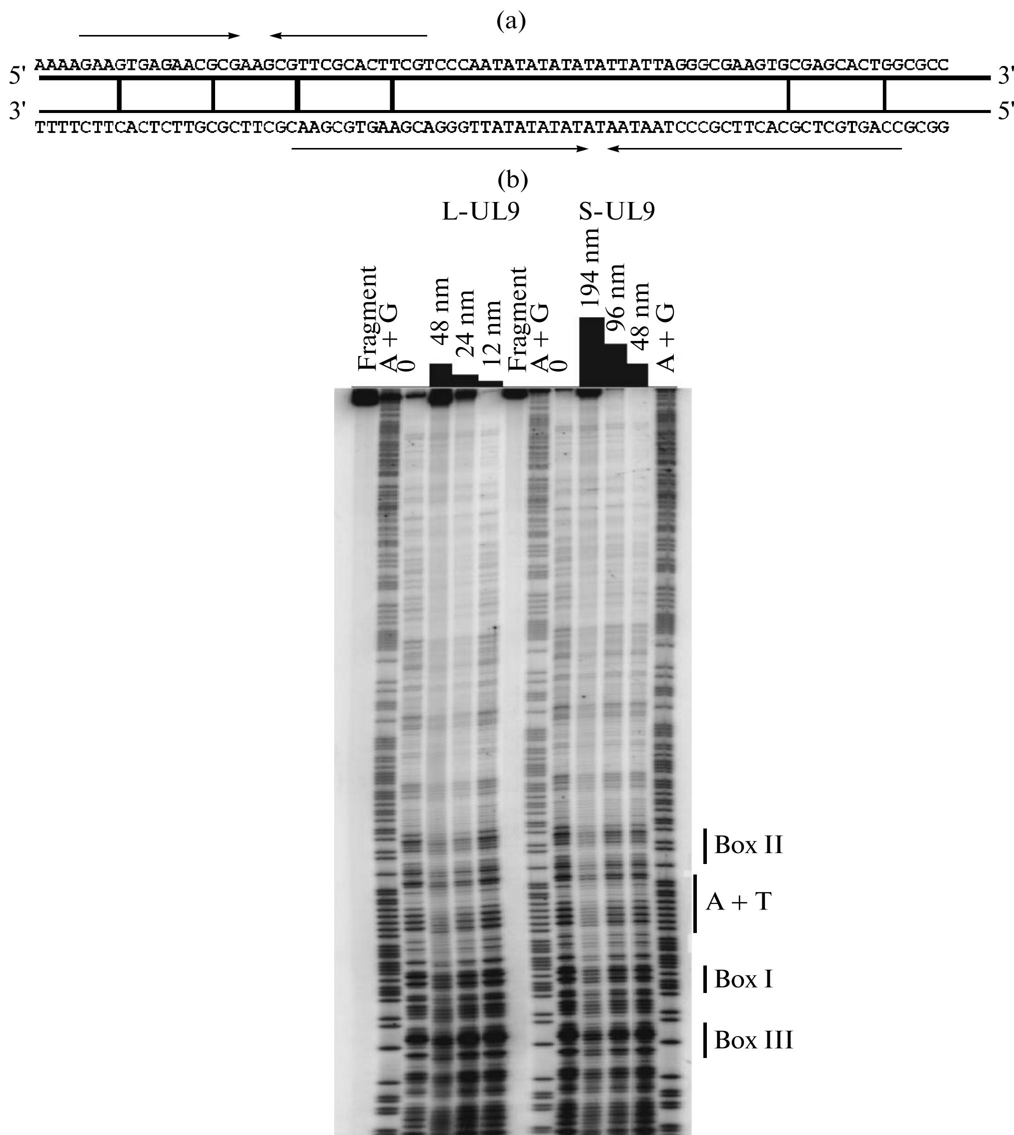


Fig. 3. Diagrams of DNase footprinting for complexes of full-sized protein UL9 (L-UL9) and its C-terminal domain (S-UL9) with the herpesvirus DNA fragment containing the replication origin OriS. (a) Nucleotide sequence of the 63-membered duplex OriS in the DNA replication origin; (b) footprints generated in the presence of L-UL9 (left half of gel) and S-UL9 (right half of gel). Protein concentrations (nM) indicated above at every lane; control (0), DNA cleavage in the absence of initiator protein; A+G, DNA cleavage at purines. Conditions: the reaction mixture contained 10 μL of radioactively labeled DNA fragment with concentration $2 \cdot 10^{-5}$ M (basepairs) and 10 μL protein in $2 \cdot 10^{-2}$ M Tris-HCl (pH 7.0) buffer in the presence of 0.01% Tween 40; 10% glycerol; $1 \cdot 10^{-3}$ M β-mercaptoethanol; $1.2 \cdot 10^{-2}$ M $MgCl_2$ and $1 \cdot 10^{-4}$ M ATP. The reaction mixture was kept for 12 h at 0 C, and then DNase I was added to the mixture, kept for three minutes at 0°C and the hydrolysis products were analyzed with the aid of polyacrylamide gel electrophoresis.

also time-dependent increase in optical density at 260 nm of solutions containing recombinant helicase L-UL9 and duplex (e)+(f) in the presence of $MgCl_2$ and ATP (data not shown). DNA untwisting can be investigated with the aid of measuring the mobility of DNA fragments in polyacrylamide gel in the absence and in the presence of protein L-UL9. Complexes of oligonucleotides with protein possess smaller mobility than free oligonucleotides (Fig. 5). The recombinant protein L-UL9 forms string complexes with each of the two single-stranded oligonucleotides in the 63-membered duplex (a)+(b) containing the A+T cluster and specific binding sites I and III for the initiator protein L-UL9. The presence of helicase activity manifests itself in that upon incubation of helicase with radioactively labeled 63-membered duplexes in the presence of $MgCl_2$ and ATP for 60 min at 42°C during gel electrophoresis there appears a band that corresponds to the mobility of a complex of single-stranded oligonucleotide with L-UL9 helicase, while the free duplex

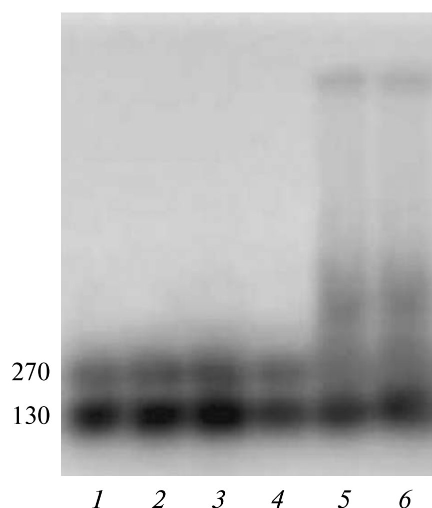


Fig. 4. Mobility of complexes of recombinant protein L-UL9 with specific (270 bp) and nonspecific DNA fragments (130 bp) in electrophoresis in 1.8% agarose gel. The OriS (400 bp) was cleaved with restriction endonuclease HindIII into two fragments that contained 270 and 130 bp respectively. Both these fragments contained a radioactive label at the 5' end, while the initial DNA fragment did not contain a radioactive label. The molar ratio of L-UL9 to the specific DMA fragment (270 bp) is equal to 1:1. Composition of reaction mixtures: lane *1* (0.04 μg 32P-DNA(270+130) + serum albumin (100 μg/mL)); lane *2* (0.04 μg 32P-DNA(270+130) + UL9); lane *3* (0.04 μg 32P-DNA(270+130) + 0.04 μg DNA(400) + UL9); lane *4* (0.04 μg 32P-DNA(270+130) + 0,08 μg DNA(400) + UL9); lane *5* (0.04 μg 32P-DNA(270+130) + 0,12 μg DNA(400) + UL9); lane *6* (0.04 μg 32P-DNA(270+130) + 0.16 μg DNA(400) + UL9). Here 32P-DNA(270+130) represents a mixture of two radioactively labeled DNA fragments, specific and nonspecific, containing 270 and 130 bp respectively.

(a)+(b) and the helicase complex with the duplex possess significantly greater mobility (Fig. 5a, lanes *5* and *6*). From the melting curve of the (a)+(b) duplex one can estimate that at a temperature of 42°C on the average three basepairs are in an untwisted state. They serve in the capacity of a "nucleus" for fluctuational opening of AT pairs and formation of a "y"-like structure, which presents a substrate for the L-UL9 helicase.

We found that the initiator protein L-UL9 forms a stronger complex with the single-stranded oligonucleotide (a) than with duplex (a)+(b) (see below). Oligonucleotide (a) in solution spontaneously folds into a hairpin stabilized by interactions with nucleotides entering boxes I and III. It is known that helicase UL9 forms a strong complex with this hairpin [12–14]. Analogous results were obtained for oligonucleotide (b), though in this case the hairpin structure is less stable. The ability of helicase L-UL9 to untwist the (a)+(b) duplex not containing a single-stranded tail at the 3' end is consistent with the data obtained for other 3'-5'-helicases. Examples may be helicases uvrD, recBCD and rep in *E. coli* cells, helicase prcA of *Bacillus stearothermofilus* and the T-antigen of SV40 [6, 7, 25, 26]. As regards the untwisting of the oligonucleotide construct (c)+(d) containing specific binding site II for the UL9 helicase, the A+T cluster and the single-stranded tail at the 3' end of oligonucleotide (c), the data of gel electrophoresis show that after incubation of duplex (c)+(d) with L-UL9 in the presence of $MgCl_2$ and ATP for 60 min at 42°C there appears a product with mobility characteristic of helicase complexes with single-stranded oligonucleotides (Fig. 5 lanes *9* and *12*). Despite that the protein preferentially binds with ssDNA, at a high ratio of protein to DNA oligomer it can interact not only with the single-stranded region of DNA but also bind at the boundary between the double-stranded and single-stranded DNA regions, which is necessary for untwisting a DNA oligomer. In accordance with the results obtained for other 3'-5'-helicases [5–7], it can be supposed that upon binding of helicase UL9 at the boundary area between double-stranded and single-stranded DNA regions the equilibrium between the double-helical and untwisted states of DNA basepairs changes, because the DNA structure deforms upon interaction with helicase in the presence of ATP. The energy of ATP hydrolysis (≈10 kcal/mol under physiological conditions) is spent not only on DNA untwisting in the boundary region but also on the translocation of the oligonucleotide along DNA.

ATP binding and ATPase activity of recombinant protein L-UL9. Presented in Fig. 6 is the curve of titration with a fluorescent analog TNP-ATP (2'/3'-O-trinitrophenyl adenosine -5'-triphosphate) of the L-UL9 protein. Fluorescence was excited at 408 nm in a one-centimeter cell, the fluorescence spectrum of free TNP-ATP had a maximum at 552 nm. The same figure presents the dependences of the intensity of fluorescence at 552 nm (I_{552}) on the ratio of the molar concentration of TNP-ATP to the concentration of L-UL9 dimers. On the curve of protein titration with the fluorescent analog TNP-ATP one observes an inflection, which points to that two molecules of the ATP analog bind with the L-UL9 dimer. The fluorescence intensity of free TNP-ATP linearly depends on its concentration in solution.

The recombinant protein possesses DNA-dependent ATPase activity. Figure 7 presents the results of chromatography on cellulose plates (Plastikfolien PEI-Cellulose F, Merck, Germany, 10 × 20 cm) of reaction mixtures containing 1.5 μL of nonspecific duplex (e)+(f) with a single-stranded fragment at the 3' end (18 nt) ($1.5 \cdot 10^{-5}$ M); 1.0 μL of radioactively labeled (gamma-^{32}P)-ATP ($1.5 \cdot 10^{-5}$ M); 1.5 μL L-UL9 ($1.5 \cdot 10^{-5}$ M) and 1 μL buffer $1 \cdot 10^{-4}$ M Tris-HCl (pH 7.2) in the presence of 50% glycerol: 20 mM $MgCl_2$; 0.05% Triton X-100; 20 mM dithiothreitol and 50 μg/μL serum albumin. In these experiments we used the oligonucleotide construct (e)+(f) that has been used by the authors of work [10] for registering the helicase activity of protein UL9 in the

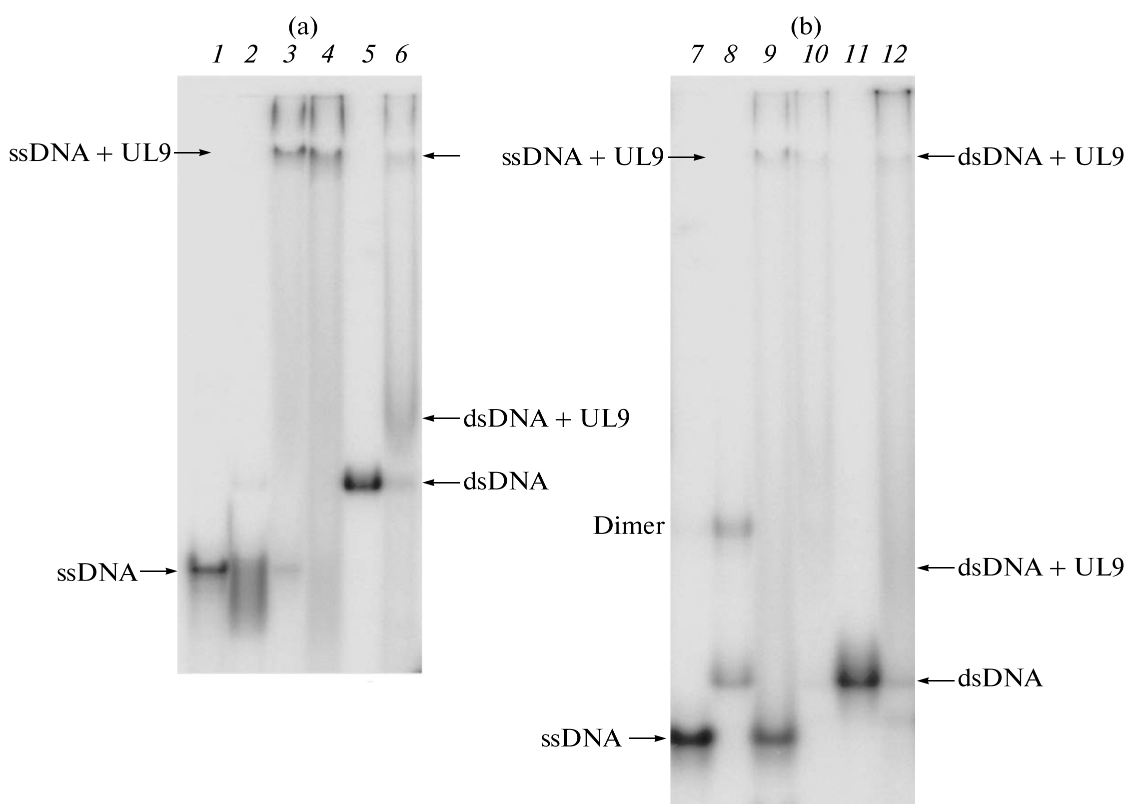


Fig. 5. Mobility of double-stranded and single-stranded 63-membered oligonucleotides OriS and OriS* and their complexes with helicase L-UL9 (Fig. 5a), and also mobilities of oligonucleotides (c) and (d), duplex (c)+(d) and complexes of these oligonucleotides with helicase L-UL9 in polyacrylamide gel electrophoresis (Fig. 5b). Lanes *1* and *2*, mobilities in polyacrylamide gel electrophoresis of free single-stranded oligonucleotides (a) and (b) corresponding to the upper and lower strands of the OriS duplex respectively. Lanes *3* and *4*, mobilities in electrophoresis of complexes of oligonucleotides (a) and (b) with protein L-UL9. Lanes *5* and *6*, mobilities in electrophoresis of free 63-membered duplex (a)+(b) and duplex (a)+(b) after incubation with helicase for 60 min in 50 mM Tris-HCl buffer (pH 7.8) in the presence of 5 mM dithiothreitol; 15 mM $MgCl_2$, 10% (vol/%) glycerol; 0.01% (vol/%) Triton X-100 and 50 mM ATP at 42°C (see section Experimental). Lanes *7* and *8*, mobilities in gel electrophoresis of oligonucleotides (d) and (c) respectively. In solution oligonucleotide (c) can exist in the form of monomer and dimer having different mobility. Lanes *9* and *10*, mobilities in electrophoresis of complexes of oligonucleotides (d) and (c) with helicase L-UL9. Lanes *11* and *12*, mobilities in electrophoresis of free duplex (c)+(d), and also duplex (c) + (d) after incubation at 42°C for 60 min with protein L-UL9 in 50 mM Tris-HCl buffer (pH 7.8) in the presence of 5 mM dithiothreitol; 15 mM $MgCl_2$; 10% (vol/%) glycerol; 0.01% (vol/%) Triton X-100 and 50 mM ATP. Electrophoresis was conducted for 2 h 10 min in 10% polyacrylamide gel (650 V, 15 W, gel temperature 22°C). Designations: ssDNA, single-stranded oligonucleotide; dsDNA, double-stranded oligonucleotide.

absence of viral protein ICP8. Reaction mixtures were kept at 42°C for 0, 10, 20, 40, 80 and 160 min and applied onto chromatographic plates. Separation was conducted for two hours in a system containing a mixture of $KHPO_4$ solution (pH 8.0) and 10% ethyl alcohol.

In Fig. 7 it is seen that the amount of radioactive phosphate cleaved off ATP increases with increasing time of enzyme incubation with ATP.

Competition of UL9 helicase and ethidium bromide for binding sites in the replication origin OriS. We investigated the binding of the dye ethidium bromide (EB) with the double-stranded duplex (a)+(b) containing the AT cluster and binding sites for helicase UL9 in the replication origin of the herpesvirus DNA (63 bp), as well as with the single-stranded oligonucleotide (a) corresponding to the upper strand of OriS duplex. Experiments were performed in the absence and in the presence of helicase L-UL9 and have shown that helicase L-UL9 and EB compete for binding sites on OriS and OriS*. If binding of EB and helicase with one and the same DNA oligomer are mutually exclusive events, then in the limit of very low levels of EB binding on the oligonucleotide the relationships (1) and (2) are fulfilled for systems containing the dye and oligonucleotide in the absence and in the presence of protein, respectively:

$$\lim_{r \to 0} r/m = K_2, \tag{1}$$

$$\lim_{r \to 0} r/m = K_2/(1 + K_1 C_1). \tag{2}$$

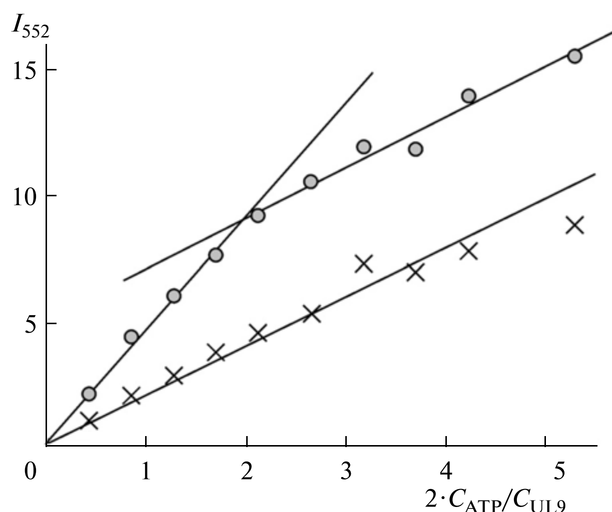


Fig. 6. Curve of titration with fluorescent ATP analog of the L-UL9 protein (circles). The initial concentration of L-UL9 dimers is equal to $6 \cdot 10^{-7}$ M . Conditions: 5 mM Tris-HCl; 5 mM HEPES-NaOH, pH 7.5; 0.025 mM EDTA; 5% (vol/%) glycerol, 0.003% Tween 40; 0,25 mM dithiothreitol, 0.24 M NaCl. Also shown is the dependence of fluorescence intensity of free TNT-ATP on its concentration (crosses). Along the abscissa axis in this case the ratio is plotted of the TNP-ATP concentration to the initial concentration of L-UL9 protein dimers.

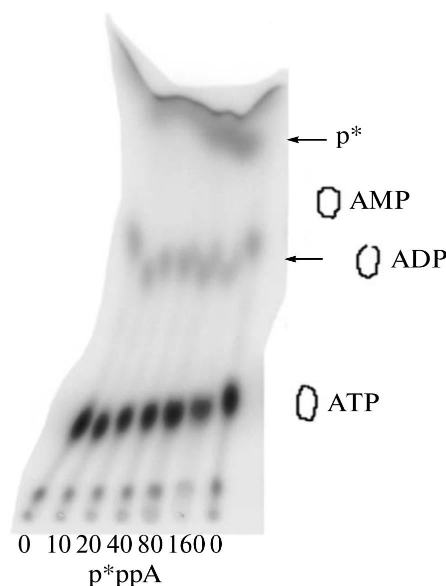


Fig. 7. Cleavage of ATP by the recombinant protein UL9. Lanes from left to right correspond to the time of incubation of the reaction mixture for 0, 10, 20, 40, 80, 160 and 0 min respectively. P* is radioactively labeled phosphate group.

Here r is the molar ratio of bound EB to oligonucleotide; K_2 is the binding constant for EB with oligonucleotide; C_1 and m are molar concentrations of free protein and EB in the examined solution.

$$\Theta = K_1C_1/(1 + K_1C_1), \quad (3)$$

$$C_t = C_1 + \Theta[O], \quad (4)$$

$$K_1 = \Theta/((1-\Theta)(C_t - \Theta[O])). \quad (5)$$

Here Θ is the extent of oligonucleotide filling with bound protein dimers; C_1 is free protein concentration; C_t is total protein concentration; [O] is duplex concentration; K_1 is the binding constant for helicase with single-stranded oligonucleotide (a) or duplex (a)+(b) containing one strong binding site for helicase.

Table 1. Cytotoxicity and antiviral activity of netropsin derivatives in cultured Vero cells infected with herpes simplex virus type I

Compound	CA_{50}	Herpes simplex virus type I		
		IC_{50}, μM	IC_{50}, μM	SI
Nt	74.5	10.0	39.7	7.5
Lys-bis-Nt	336.7	11.6	34.9	29.0
Pt-bis-Nt	258.3	4.4	8.7	59.0
Pt*-bis-Nt	98.9	>100	>100	0.0
15Lys-bis-Nt	60.4	1.3	3.4	47.0

Note: CA_{50}, concentration at which 50% cell death is observed in noninfected cell culture. IC_{50} and IC_{95}, concentrations causing 50% and 95% protective effect in Vero cell cultures infected with herpesvirus. SI, selectivity index.

Figure 8 gives the adsorption isotherms for EB on the 63-membered double-stranded and single-stranded oligonucleotides OriS and OriS* in the absence and in the presence of helicase L-UL9.

From the curves of fluorimetric titration with EB of OriS and OriS* in the absence and in the presence of protein we have determined the constants of equilibrium binding of UL9 helicase with OriS and OriS*: $K_1 = 1.8 \cdot 10^7$ M^{-1} for protein binding with the 63-membered duplex OriS; upon binding with single-stranded oligonucleotide (upper strand in OriS duplex) $K_1 = 1.5 \cdot 10^8$ M^{-1}. The initiator protein L-UL9 forms a stronger complex with single-stranded oligonucleotide (a) than with duplex (a)+(b). In conducting calculations we used formulae (1)–(5).

Antiviral activity of bis-netropsin. In our previous works we have studied the DNA-binding and antiviral activity of a series of bisNt in which two monomers are connected by various linkers [15–18, 21, 27–29]. It is shown that some of these compounds possess high antiherpetic activity and low cytotoxicity as compared with antibiotic Nt and its dimeric analogs investigated by the authors of work [30]. The antiviral activity of Nt derivatives may be caused by that they inhibit the thermal fluctuation-caused opening of AT basepairs in the A+T cluster in OriS and prevent the formation of a DNA bend induced upon binding of UL9 with specific binding sites I and II in OriS. The single-stranded

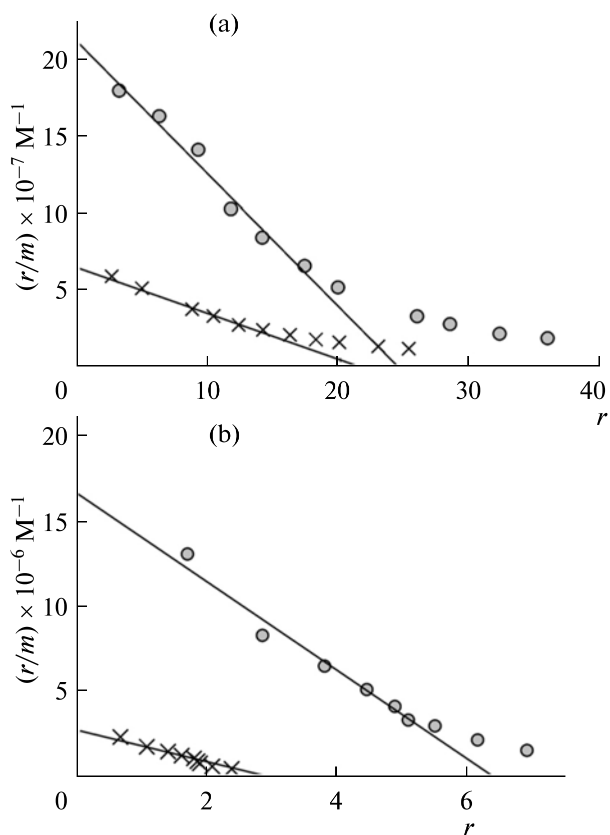


Fig. 8. (a) Adsorption isotherms for dye ethidium bromide on 63-membered duplex OriS in Scatchard coordinates in the absence (circles) and in the presence (crosses) of helicase L – UL9; *r* is molar ratio of bound EB to duplex; *m* is concentration of free EB. Duplex concentration equals $2.27 \cdot 10^{-7}$ M, L – UL9 dimer concentration equals $2.83 \cdot 10^{-7}$ M. (b) Adsorption isotherms for dye ethidium bromide on single-stranded 63-membered oligonucleotide OriS* in Scatchard coordinates in the absence (circles) and in the presence (crosses) of L-UL9; *r* is molar ratio of bound EB to OriS*; *m* is concentration of free EB. Oligonucleotide concentration equals $1.6 \cdot 10^{-7}$ M, L-UL9 dimer concentration equals $1.7 \cdot 10^{-7}$ M. Conditions: 2.5 mM Tris-HCl; 2.5 mM Na-HEPES, pH 7.5; 0.14 M NaCl; 0.01 mM EDTA; 2% (vol/%) glycerol; 0.001% Tween 40; 0.1 mM dithiothreitol.

63-membered oligonucleotide corresponding to the upper strand in the OriS duplex spontaneously folds into two hairpins—GC-rich hairpin formed at the expense of nucleotide pairing in boxes I and III, and short AT-rich hairpin melting at low temperature (35°C) (Fig. 9a). Helicase forms a strong complex with the GC-rich hairpin.

We have disclosed that upon binding of the 63-membered oligonucleotide (a)+(b) with bisNt there is an increase in the melting temperature (T_1) of the short AT-rich hairpin and practically no change in the melting temperature (T_2) of the long GC-rich hairpin (see Table 2).

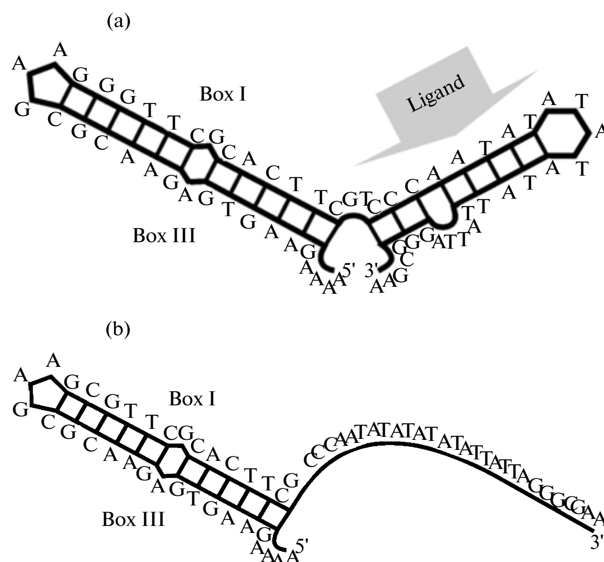


Fig. 9. Hairpin structures formed by the upper strand in OriS; (a) stabilization by a DNA-binding ligand of the structure of an AT-rich hairpin; (b) active form used in the process of viral DNA replication, contains a GC-rich hairpin and extended unstructured tail at the 3' end.

Stabilization of the structure of the AT-rich hairpin by bisNt hinders formation of an active intermediate structure of OriS, an important property of which is the presence instead of the AT-rich hairpin of an extended unstructured "tail" at the 3' end (≈18 nt) (Fig. 9b). The antiviral activity of bisNt is probably associated with that they suppress two important stages in the process of DNA untwisting by helicase UL9—fluctuational opening of AT pairs in the A+T cluster in the replication origin and the process of formation of an extended unstructured tail at the 3' end.

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Table 2. Melting temperatures of AT- and GC-rich hairpins in oligonucleotide (a) in the absence and in the presence of bis-netropsins

Preparation	T_1, °C	T_2, °C
O1 (upper strand)	35	82
O1 + Lys-bis-Nt	57	84
O1 + Pt-bis-Nt	47	83
O1 + Pt*-bis-Nt	45	84
O1 + 15Lys-bis-Nt	63	84

Note: O1–63-membered single-stranded oligonucleotide (a).

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