Volume 234, number 1, 65–68 FEB 05999 July 1988

Translation arrest of potato virus X RNA in Krebs-2 cell-free system: RNase H cleavage promoted by complementary oligodeoxynucleotides

Nadezhda A. Miroshnichenko, Olga V. Karpova, Sergei Yu. Morozov, Nina P. Rodionova and Joseph G. Atabekov

Institute of Microbiology, Moscow 117811 and Department of Virology, Moscow State University, Moscow 119899, USSR

Received 26 April 1988

Translation arrest of genomic potato virus X (PVX) RNA promoted by complementary oligodeoxynucleotides in Krebs-2 cell-free system is described. 14-15 mer oligodeoxynucleotides complementary to the 5′-proximal cistron of PVX RNA were shown to induce specific truncation of the major non-structural polypeptide coded by PVX RNA. Evidence is presented that effective translational arrest of PVX RNA in the presence of complementary oligonucleotides restults from the site-specific cleavage of RNA by endogenous RNase H intrinsic to the Krebs-2 extract. No similar translational arrest was found in the rabbit reticulocyte lysate cell-free system.

Translational arrest; Cell-free system; RNase H cleavage; Plant virus RNA

1. INTRODUCTION

It has been shown that mRNA translation can be specifically arrested by a complementary DNA fragment or oligodeoxynucleotide both in vitro and in vivo [1–4]. Since the early in vitro studies the translational arrest has been assumed to be due to the blocking of ribosome translocation on the appropriate mRNA resulting from RNA-DNA hybrid formation [1,2]. However, it has been demonstrated that RNA cleavage due to an endogenous RNase H activity both in wheat germ extract and in *Xenopus* oocytes may be responsible for hybrid arrest of translation by complementary DNAs [5–7].

Potato virus X (PVX) is a type member of the potexvirus group. The complete nucleotide sequence (6435 bases) of the genomic PVX RNA has been determined recently [8]. PVX RNA directs in vitro the major non-structural protein of 165 kDa corresponding to the 5′-proximal open reading frame coding for a putative component of the viral RNA replicase [8].

Here we consider the site-specific arrest of translation of PVX RNA in Krebs-2 ascite tumor extract which occurs upon addition of oligodeoxynucleotides complementary to defined coding sequences within the 5′-proximal 165 kDa gene.

Correspondence address: N.A. Miroshnichenko, Institute of Microbiology, Moscow 117811 USSR

2. MATERIALS AND METHODS

PVX, wild strain, was propagated on *Datura stramonium* L. plants. The virus preparations and virion RNA were obtained as described [9]. Ribonuclease H (RNase H) was isolated from *Escherichia coli* MRE-600 as described by Rodionova et al. [10]. Hybrid formation and RNase H cleavage reactions were carried out as described [11] with slight modifications. Reaction mixtures were incubated for 3 h at 4°C. The activity of RNase H was determined using [^{14}C]RNA-DNA hybrid as a substrate [12]. Oligonucleotide 5′d[GTTTTGGAATGTCTC]-oligo I was synthesized and kindly provided by B.K. Chernov. Oligonucleotide 5′d[CAAGAAGAGCTGCA]-oligo II was synthesized and kindly provided by T.S. Oretskaya. The procedures of cell-free translation in wheat germ, rabbit reticulocyte lysate or Krebs-2 cell-free systems have been described [13–16]. Preincubation of PVX RNA (5 mg) and RNA-DNA hybrids (5 mg RNA with 0.01 A_{260} units of the

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50 © 1988 Federation of European Biochemical Societies

desired oligonucleotide) in the Krebs-2 translation mixture (50 ml) was carried out at 30°C for 30 min. RNA was extracted with phenol, precipitated with ethanol and dissolved in 20 ml of water. Samples (5 ml) were translated in standard reticulocyte lysate. Reticulocyte lysate supplemented with RNase H contained 0.02 units of *E. coli* RNase H in 25 ml sample. [^{35}S]Methionine-labelled proteins from cell-free translation mixtures were analyzed by gel electrophoresis in 8–20% SDS-polyacrylamide gel as described [13]. For blot-hybridization analysis RNA samples were re-extracted from Krebs-2 translation mixture, denatured with formaldehyde, electrophoresed in 1.5% agarose gels, transferred to nitrocellulose and hybridized to [^{32}P]cDNA probe (1000 bases) specific for the 5′-proximal region of the PVX genome [17].

3. RESULTS AND DISCUSSION

In a separate series of experiments it was demonstrated that PVX RNA can be specifically cleaved by RNase H from *E. coli* in the presence of two synthetic oligodeoxynucleotides: 15-mer (oligo I) complementary to nucleotides 562–576 and 14-mer (oligo II) complementary to nucleotides 1022–1035 from the 5′-end of PVX RNA (not shown).

The 5′-proximal cistron of PVX RNA codes for a non-structural 165 kDa polypeptide, presumably RNA polymerase [8]. Therefore, the site-specific cleavage of PVX RNA by RNase H directed by oligo I and oligo II should release the 5′-terminal capped RNA fragments coding for the N-terminal parts of the 165 kDa protein. This suggestion is supported by the data obtained with the use of the Krebs-2 translation system (shown in fig.1). The native genomic PVX RNA directs the synthesis of the 165 kDa protein (fig.1E and F). Polypeptides of 19 kDa (fig.1D) and 35 kDa (fig.1B) were programmed by genomic RNA cleaved in the presence of oligo I and oligo II, respectively. It is of importance that polypeptides of the same sizes were also synthesized in translational systems containing the hybrids of PVX RNA with oligonucleotides I or II which were not pretreated with exogenous RNase H (fig.1A and C). These polypeptides obviously arise from the translation initiation on the capped 5′-fragments of PVX RNA since their production can be completely inhibited by the cap-analogue ^{7}mGDP (not shown). Hence, the resulting truncated 19 kDa and 35 kDa products should represent the N-terminal parts of the 165 kDa polypeptide.

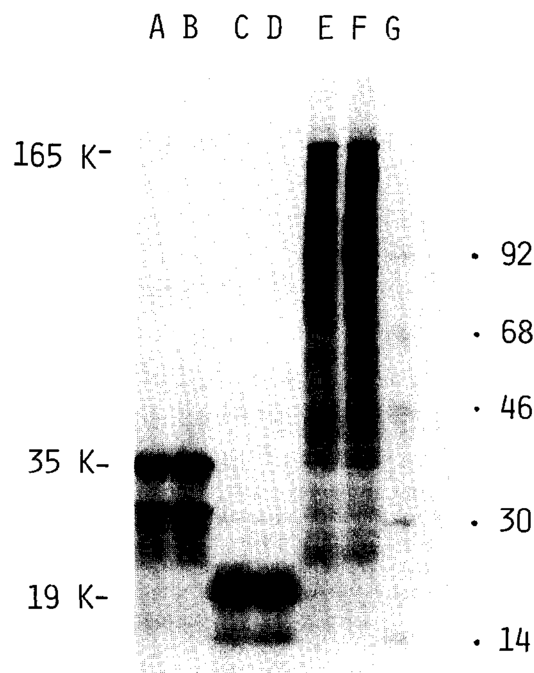

Fig.1. Translational arrest of PVX RNA in Krebs-2 cell-free system mediated by oligodeoxynucleotides complementary to the 5′-proximal gene coding for 165 kDa protein. Translation products of PVX RNA preincubated with: oligo II (A); oligo II + *E. coli* RNase H (B); oligo I (C); oligo I + RNase H (D); no oligonucleotide added (E); RNase H without oligonucleotide (F). Molecular mass of protein standards (G) is indicated in kDa (right). The positions of the major in vitro products are shown (left).

Similar experiments with the use of rabbit reticulocyte lysate revealed that oligonucleotides I and II did not block the translation of PVX RNA (fig.2C and E). The specific arrest of translation in the presence of these oligonucleotides has been observed only in the case of direct addition of *E. coli* RNase H to lysate (fig.2D and F).

Two enzymes account for the RNase H activities in Krebs-2 ascites tumour cells [18]. To verify whether the Krebs-2 extract contained enough RNase H activity to digest RNA-DNA hybrids, the PVX RNA was incubated in the presence of oligonucleotides I or II in Krebs-2 system under translation conditions. Then RNA was extracted and analyzed by Northern blot hybridization using a radioactive cDNA-probe specific for the 5′-terminal 1.0 kb region of the PVX genome. Two 5′-terminal RNA fragments of about 0.6 and

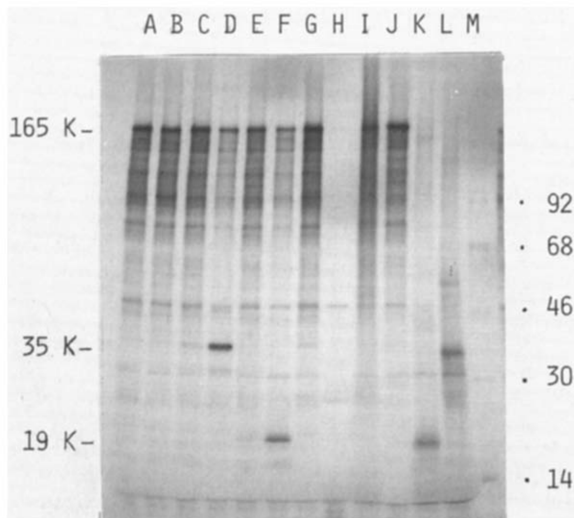

Fig.2. PVX-specific translation in rabbit reticulocyte lysate. Translation products of PVX RNA preincubated with: no oligonucleotide (control) (A,B,G); oligo II (C,D); oligo I (E,F); no oligonucleotide + Krebs-2 extract (I); TMV-specific oligonucleotide + Krebs-2 extract (J); oligo I + Krebs-2 extract (K); oligo II + Krebs-2 extract (L). Samples B, D and F contained *E. coli* RNase H. Sample H had no RNA added. Molecular mass of protein standards (M) is indicated in kDa (right). The positions of the major in vitro products are shown (left).

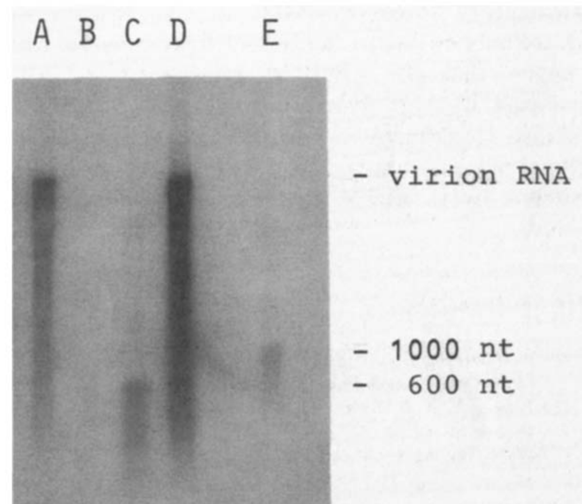

Fig.3. The site-specific cleavage of PVX RNA in Krebs-2 extract promoted by complementary oligodeoxynucleotides. Blot-hybridization analysis of PVX RNA preincubated in Krebs-2 extract with: 11-mer TMV-specific oligonucleotide (control) (A); oligo I (C); no oligonucleotide added (D); oligo II (E). No PVX RNA added (B) (control). The positions of hybridizing species are indicated.

1.0 kb long were cleaved from the PVX RNA in Krebs-2 extract in the presence of oligo I and oligo II, respectively (fig.3C and E). The resulting fragments comigrated with the products of site-specific cleavage of PVX RNA by *E. coli* RNase H (not shown). These PVX RNA fragments directed the synthesis of the polypeptide of 19 kDa (0.6 kb fragment) and the polypeptide of 35 kDa (1.0 kb fragment) in rabbit reticulocyte lysate (fig.2K and L). The presence of the minor high molecular mass products in fig.2K and L might be due to the translation of the 3′-terminal fragments of PVX RNA. Genomic PVX RNA re-extracted after preincubation in Krebs-2 extract alone or in the presence of heterologous (TMV-specific) oligonucleotide remained intact (fig.3A and D) and was efficiently translated in reticulocyte lysate into major product – the 165 kDa PVX-specific polypeptide (fig.2I and J).

As follows from table 1, the presence of RNase H activity is characteristic of wheat germ extract as has been reported by other groups [5,6]. RNase H activity can also be detected in Krebs-2 extract, while it is not detected in rabbit reticulocyte lysate. Earlier it was postulated that an 'unwinding activity' associated with reticulocyte ribosomes may remove the DNA fragment or oligonucleotide from the heteroduplex upon the translation elongation [19]. Our data suggest that the absence of translation arrest of the PVX RNA by com-

Table 1

RNase H activity in cell-free translational systems

Incubation with	Trichloroacetic acid-soluble fraction (cpm/sample)[a]	Relative activity (%)
RNase H from *E. coli*	3000	100
Krebs-2 extract	947	32
Wheat germ extract	2647	85
Reticulocyte lysate	55	–
Control[b]	65	–

[a] [^{14}C]RNA-DNA hybrid (7000 cpm) was incubated with 50 ml of translational extract or with *E. coli* RNase H (0.02 units) for 60 min at 30°C. Radioactivity of trichloroacetic acid-soluble fraction was determined

[b] [^{14}C]RNA-DNA hybrid was incubated alone as described [12]

plementary oligonucleotides in the reticulocyte lysate may be due to the lack of RNase H. We also suggest that the arrest of translation, both in eukaryotic cells which possess an endogenous RNase H activity and in cell-free extracts from these cells, is due to the site-specific cleavage of mRNA by RNase H directed by complementary DNA.

REFERENCES

[1] Paterson, B.M., Roberts, B.E. and Kuff, E.L. (1977) Proc. Natl. Acad. Sci. USA 74, 4370–4374.

[2] Blake, K.R., Murakami, A. and Miller, P.S. (1985) Biochemistry 24, 6132–6138.

[3] Haeuptle, M.T., Frank, R. and Dobberstein, B. (1986) Nucleic Acids Res. 14, 1427–1446.

[4] Kawasaki, E.S. (1985) Nucleic Acids Res. 13, 4991–5004.

[5] Minshull, J. and Hunt, T. (1986) Nucleic Acids Res. 14, 6433–6451.

[6] Cazenave, C., Loreau, N., Thuong, N.T., Toulene, J.J. and Helene, C. (1987) Nucleic Acids Res. 15, 4717–4736.

[7] Dash, P., Lotan, I., Knapp, M., Kandel, E.R. and Goelet, P. (1987) Proc. Natl. Acad. Sci. USA 84, 7896–7900.

[8] Krayev, A.S., Morozov, S.Yu., Lukasheva, L.I., Rosanov, M.N., Chernov, B.K., Simonova, M.L., Golova, Yu.B., Belzhelarskaya, S.N., Pozmogova, G.E., Skryabin, K.G. and Atabekov, J.G. (1988) Dokl. Akad. Nauk SSSR 301, in press.

[9] Guilford, P.J. and Forster, R.L.S. (1986) J. Gen. Virol. 67, 83–90.

[10] Rodionova, N.P., Karpova, O.V., Metelev, V.G., Bogdanova, S.L., Shabarova, Z.A. and Atabekov, J.G. (1983) Mol. Biol. (Moscow) 17, 809–817.

[11] Tyulkina, L.G., Karpova, O.V., Rodionova, N.P. and Atabekov, J.G. (1987) Virology 159, 312–320.

[12] Metelev, V.G., Stepanova, O.B., Chickova, N.V., Smirnov, V.D., Rodionova, N.P., Berzin, V.M., Jensone, I.V., Gren, E.J., Bogdanov, A.A., Shabarova, Z.A. and Atabekov, J.G. (1980) Mol. Biol. (Moscow) 14, 200–211.

[13] Karasev, A.V., Alexandrova, N.M., Miroshnichenko, N.A., Nesterova, I.V. and Dorokhov, Yu.L. (1987) Mol. Biol. (Moscow) 21, 1367–1377.

[14] Roberts, B.E. and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330.

[15] Ugarova, T.Yu. (1977) in: Modern Methods in Biochemistry, vol.3, pp.358–368, Medicina, Moscow.

[16] Karasev, A.V., Miroshnichenko, N.A. and Ugarova, T.Yu. (1988) Mol. Biol. (Moscow) 22, in press.

[17] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[18] Cathala, G., Rech, J., Huet, J. and Jeanteur, Ph. (1979) J. Biol. Chem. 254, 7353–7361.

[19] Liebhaber, S.A., Cash, F.E. and Shakin, S.H. (1984) J. Biol. Chem. 259, 15597–15602.