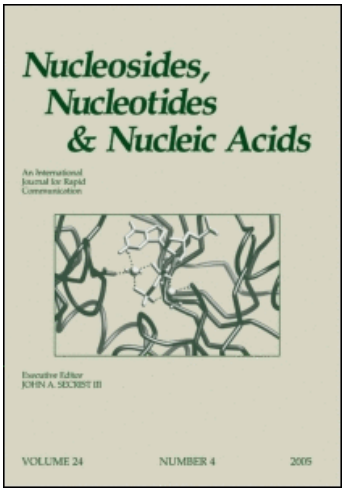

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THE EFFECTS OF 2-5A ON PROTEIN SYNTHESIS IN WHEAT GERM EXTRACTS AND TOBACCO PROTOPLASTS

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Abstract. Nonphosphorylated 2-5A inhibited translation and caused RNA degradation in wheat germ extract, whereas 3-5A had no effect. Protein synthesis inhibition by 2-5A was observed in tobacco protoplasts. 70 kD 2-5A-binding protein was found in potato leaf extracts by chemical crosslinking.

Interferons (IFNs) are antiviral proteins secreted by animal cells in response to not only viral infection, but also cell proliferation and various immunological processes.[1] The 2'-5' oligoadenylate pathway is also affected by IFN. IFN binding to cell surface receptors induces the synthesis of 2'-5' oligoadenylate synthetase, which in the presence of double-stranded RNA polymerizes ATP to form a series of unique 2'-5' oligoadenylates (2-5A). 2-5A trimers and longer oligomers bind to and activate a latent endoribonuclease (RNase L), which leads to the inhibition of protein synthesis by degrading mRNA. Some components of the 2-5A pathway have been detected in plants. Devash *et al.* have demonstrated that 2-5A can inhibit tobacco mosaic virus (TMV) replication in tobacco protoplasts, leaf discs, and intact plants. 2-5A trimer "cores" are the most potent inhibitors.[2,3] On the basis of these data Devash *et al.* postulated that a system analogous to the mammalian 2-5A pathway may exist in higher plants. Two glycoproteins (gp22 and gp35), which production is stimulated by virus infection, were purified from plants and found to cross-react with human β-interferon polyclonal antibodies.[4] Later, they were identified as an isoform of the pathogenesis-related protein 5 and

β-1,3-glucanase.[5] Reichman *et al.* reported ATP-polymerizing activity in antiviral factor-treated leaves of *Nicotiana glutinosa* giving rise to plant oligonucleotides with antiviral activity.[6] Later Devash *et al.* demonstrated the poly(rI)· poly(rC)-dependent synthesis of oligoadenylates in tobacco leaves and cell cultures.[7] They claimed, however, that these oligoadenylates differed substantially from the mammalian 2-5A.[7] Recently Kulaeva *et al.* have shown that human IFN and 2-5A increase the cytokinin content and induce the synthesis of various proteins in plant cells.[8] However, IFN did not appear to inhibit the replication of turnip yellow mosaic virus and alfalfa mosaic virus.[9-11] DNA sequences homologous to human 2-5A synthetase were found in tobacco genomic DNA and a 2-5A synthetase was purified from tobacco and found to cross-react with human 2-5A synthetase antibodies.[12] We have not detected the sequences hybridizing to rat 2-5A synthetase cDNA in tobacco and potato mRNA or genomic DNA.[13,14] But we have found 2-5A-degrading activity in tobacco leaf extracts.[14] Little is known about other 2-5A-binding proteins of the plant 2-5A pathway. Devash *et al.* could not detect (2'-5')p_3A_4[^{32}P]pCp-binding proteins in *N. glutinosa* leaves.[7] As plant oligoadenylates could not activate RNase L from rabbit reticulocyte lysates (RRLs) and did not compete with (2'-5')p_3A_4[^{32}P]pCp for binding to mammalian RNase L, Devash *et al.* concluded that the oligoadenylate-dependent protein synthesis inhibition in plants occurs without activating a 2-5A-dependent RNase.[7] Cayley *et al.* could not detect 2-5A-binding proteins in tobacco either.[15]

Mammalian 2-5A-synthetase has recently been expressed in transgenic plants and provides protection against several plant RNA viruses.[13,14,16,17] These data indicate that 2-5A-binding protein(s) may exist in plant cells. In this study we report for the first time the presence of a 2-5A-binding protein in potato leaf extracts. We show that the 2-5A trimer "core" is the most potent inhibitor of translation in a plant cell-free system and *in vivo* in tobacco protoplasts.

MATERIALS AND METHODS

Purification of 2-5A oligomers

2'-5' and 3'-5' oligoadenylates, chemically synthesized by Prof. I. A. Mikhailopulo, Institute of Bioorganic Chemistry, Minsk, Belarus, as

nonphosphorylated "core" forms, were finally purified by HPLC.[18] Synthesis of the mono-, di- and triphosphate forms was performed by Prof. Mikhailopulo's group by sequential addition of 5' phosphate groups to the "core" molecules.[19] 2-5A trimers and tetramers with differing degrees of phosphorylation were purified with HPLC on amino-Sil-X-1 columns (Tessek, Czechia) using a DuPont 8810 chromatograph with UV detector. A concentration gradient of buffer A (20% acetonitrile in 6.25 mM K-phosphate buffer, pH 6.45) and buffer B (13% acetonitrile with 0.6 M $LiClO_4$ in 6.25 mM K-phosphate buffer, pH 6.45) was used with a gradient of 50% to 100% of buffer B in 15 min. Samples were desalted by lyophilization and stored at -20°. Prior to use the samples were dissolved in sterile RNase-free double-distilled water.

In addition, we used independently synthesized 2-5A trimers (a mixture of oligomers with varying degrees of phoshorylation) provided by Prof. W. E. G. Müller, University of Mainz, Germany. These oligomers were treated with calf intestine alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's instructions. The resulting nonphosphorylated trimers were purified on HPLC using Supelcosil LC-18 column (Supelco) in a concentration gradient of buffer A (0.1 M triethylammoniumacetate, pH 7) and buffer B (0.1 M triethylammoniumacetate in 40% acetonitrile) with a gradient of 10% to 30% of buffer B in 30 min. Samples were lyophilized, stored, and dissolved as described above.

ATP, AMP and adenosine were purchased from Sigma.

In vitro *translation assays*

Assays in wheat germ extract (WGE, Amersham or Promega) and rabbit reticulocyte lysate (RRL, Amersham) were carried out according to manufacturers' instructions, using 0.8 μg of TMV RNA (prepared from TMV particles according to[20]) or 0.5 μg of brome mosaic virus (BMV) RNAs (Promega) and 5 μCi (WGE) or 3.5 μCi (RRL) of DL-[4,5-^{3}H]Leu (34 Ci/mmol, Amersham) or 10 μCi of L-[^{35}S]Met (>1000 Ci/mmol, Amersham) per reaction (30 μl). Samples (5 μl) were collected on Whatman GF/C filters, incubated on ice 5 min with cold 5% trichloroacetic acid (TCA), heated 10 min at 90°C, incubated again on ice for 5 min with cold TCA, washed sequentially with 30% H_2O_2 (omitted for WGE), water, ethanol, and acetone and then

dried with a warm air blower. The dried filters were counted using a toluene-based scintillant on LKB Wallac Rackbeta 1215 scintillation counter. BMV RNA translation products were separated by 12% PAGE in the Laemmli system[21], gels dried and autoradiographed. Autoradiographs were scanned with an LKB 2202 UltroScan laser densitometer to quantify the amount of synthesized proteins.

Covalent binding of [^{32}P]pCp-labeled 2-5A to proteins in cell extracts

Nonphosphorylated HPLC-purified 2-5A and 3-5A tetramers were ligated to cytidine-3',5'-[5'-^{32}P]-bisphosphate (pCp, 3000 Ci/mmol, Amersham) following the method described by Knight *et al.*[22] and A_4[^{32}P]pCp purified in an HPLC system using a reverse phase ODC C18 column (DuPont) according to.[23] The 3'-phosphate was removed by *Escherichia coli* alkaline phosphatase type III (Sigma) according to[24] and the dephosphorylated products purified by HPLC on an ODC C18 column. The chemical crosslinking procedure was carried out essentially as described by Wreschner *et al.*[24] UV crosslinking was done essentially as described by Nolan-Sorden *et al.*,[25] performing the irradiation in Stratalinker (Stratagene) twice at maximum energy at the distance of 6 cm from the bulbs. Extracts of potato (*Solanum tuberosum*) leaves, wheat germ, mouse L-cells, and mouse spleen were used as the sources of oligoadenylate-binding proteins. The binding specificity of labeled 2-5A to proteins was controlled in competition assays, by adding excess (100 μM) unlabeled 2-5A and 3-5A to the reaction mixtures. The reaction mixtures were separated on 12% SDS-PAGE as described above.

RNA degradation analysis

Samples were taken from *in vitro* translation mixtures after 0, 15, and 30 min incubation and frozen in liquid nitrogen. RNA was isolated from the samples as described by us earlier,[26] analyzed by electrophoresis in 1% agarose/formamide gels and Northern blotted to Hybond™ nylon membranes according to Amersham protocols. ^{32}P-labeled cDNA was synthesized on TMV RNA templates according to[27] using hexanucleotide random primers and [^{32}P]dNTP (4000 Ci/mmol, Radiopreparat, Uzbekistan). To quantify the RNA degradation, autoradiographs were scanned by laser densitometer as described above.

Protein synthesis in tobacco protoplasts

Protoplasts from *N. tabacum* SR1 leaves (grown on MS[28] agar medium at 24°C during a 16 h photoperiod) were obtained according to[29], except that after enzyme treatment protoplasts were washed and maintained in K3 medium[30] containing 400 mM sucrose. 1 μCi of ^{14}C-labeled protein hydrolysate (0.5 Ci/milliatom C, Chemapol, Czechia) per 10^6 protoplasts was added. Samples were collected after 0, 30, 60, 90, and 120 min incubation and frozen in liquid nitrogen. After homogenization with half a volume of 100 mM Tris-HCl pH 7.8, proteins were precipitated with three volumes of ice-cold 10% TCA for 30 min and collected on Schleicher & Schuell No. 6 filters. The filters were dried and counted in toluene-based scintillant. To ensure that the 2-5A "core" does not inhibit the uptake of amino acids by tobacco protoplasts, a control experiment was carried out. In the control, besides the ^{14}C-labeled protein hydrolysate, cycloheximide at a final concentration of 1 mM was added to prevent *de novo* protein synthesis. After a 30 min incubation, the cells were washed three times with growth medium and total cellular radioactivity was counted in a dioxane-based scintillant.

RESULTS

Purification of 2-5A oligomers

The separation of 2-5A oligomers having the same chain length but different degrees of phosphorylation was achieved with HPLC using a weak anionite amino-Sil-X-1 columns and a lithium perchlorate gradient in volatile buffer (FIG. 1). The purity of individual 2-5A oligomers was greater than 99%. The purity of "core" forms, obtained from the Institute of Bioorganic Chemistry, Minsk was checked on HPLC in methanol gradient according to Brown *et al.*[23] It was always higher than 99%. 2-5A trimers from the University of Mainz, after the enzymatic dephosphorylation and HPLC purification, had the same degree of purity.

The effect of 2-5A, 3-5A, ATP, AMP, and adenosine on TMV RNA translation in WGE and RRL

The effects of various 2-5A derivatives, 3-5A tetramers, ATP, AMP, and adenosine on the cell-free translation of TMV RNA in WGE and RRL are presented in TABLE 1. The 2-5A trimer "core" (A_3) and tetramer "core"

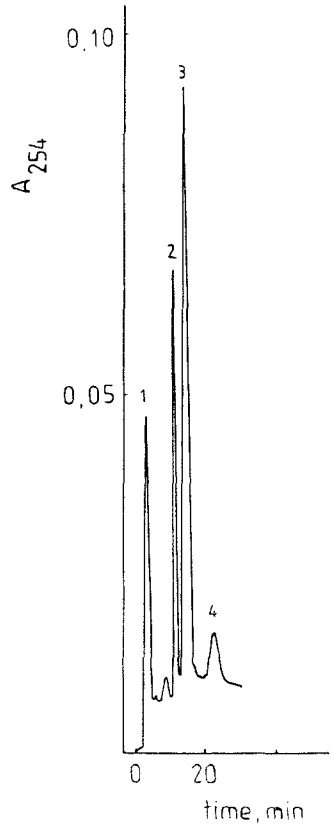

FIG. 1. Purification of 2-5A trimers with HPLC. Column (3.5 x 140 mm) with amino-Sil-X-1, sample volume was 20 µl. 1 - p_3A_3, 2 - p_2A_3, 3 - pA_3, 4 - A_3.

(A_4) were the most potent inhibitors among the 2-5A derivatives. The inhibitory effects of the phosphorylated 2-5A trimers pA_3, ppA_3, and $pppA_3$ were significantly lower than that of the 2-5A trimer "core". The same is true for 2-5A tetramer derivatives (TABLE 1). Unlike the triphosphorylated trimers, 200 nM $pppA_4$ activated TMV RNA translation in WGE. Micromolar concentrations of 3-5A tetramer "core", ATP, AMP, and adenosine had no effect on TMV RNA translation in WGE. No differences were observed in translation inhibition rates when 2-5A trimer "cores" provided either by Prof. Mikhailopulo or Prof. Müller were used (data not shown).

TABLE 1. The inhibition of TMV RNA translation in WGE and RRL by 2-5A, 3-5A, ATP, AMP, and adenosine. The translation rate in WGE and RRL in the presence of 2-5A tri- and tetramers with the different phosphorylation, 3-5A tetramers, ATP, AMP, and adenosine is shown as % of that without 2-5A (positive control).

Added nucleotide	WGE[a]	RRL[a]
positive control	100	100
3 μM adenosine	102.5 ± 11.9	nd[b]
3 μM AMP	108.2 ± 16.9	nd
3 μM ATP	104.6 ± 15.0	nd
1 μM 3'-5' A_4	100.3 ± 9.2	nd
1 μM A_3	27.4 ± 7.4	93.6 ± 4.6
1 μM pA_3	92.3 ± 7.3	95.9 ± 2.8
1 μM ppA_3	66.9 ± 4.5	78.4 ± 5.3
1 μM $pppA_3$	87.9 ± 6.6	63.1 ± 5.4
200 nM A_4	62.5 ± 3.6	81.0 ± 7.0
200 nM pA_4	82.0 ± 4.3	63.0 ± 8.1
200 nM ppA_4	84.5 ± 9.9	55.0 ± 12.1
200 nM $pppA_4$	121.6 ± 2.3	44.8 ± 11.3

[a]The samples were taken from the *in vitro* translation mix and the incorporation of [^{3}H]-leucine into protein was measured as described in Materials and Methods.
[b]Not determined.

In RRL, the most potent inhibitors of protein synthesis, as expected, were triphosphorylated oligoadenylates. The tetramer triphosphate showed a greater inhibitory effect than corresponding trimer (TABLE 1). The concentration dependence of 2-5A trimers and tetramer "cores" on the inhibition of WGE protein synthesis was studied separately. Micromolar concentrations of the 2-5A trimer and tetramer "cores" were necessary for the inhibitory effect (FIG. 2 A and B).

2-5A tetramer "core" also inhibits BMV RNA *in vitro* translation in WGE.In the presence of 1 μM A_4 "core" the amount of four major BMV translation products is reduced at least five-fold when compared to the

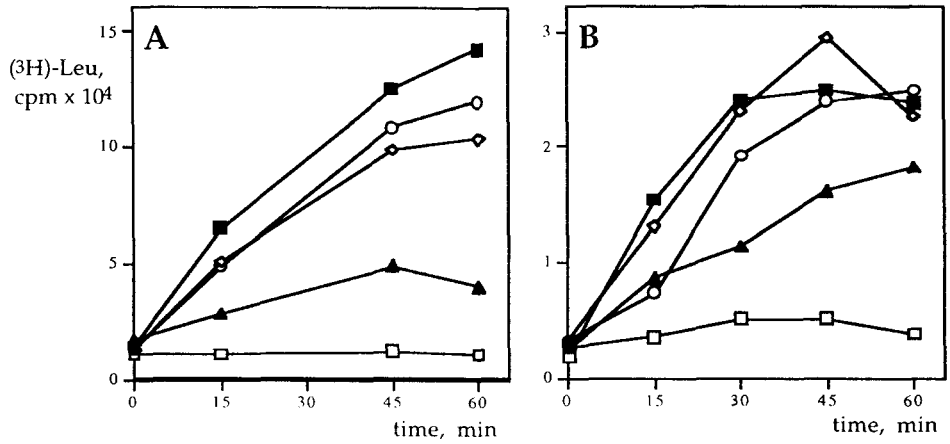

FIG. 2. The inhibition of TMV RNA translation in WGE by 2-5A. The dependence of inhibition on 2-5A trimer (A) and tetramer (B) "core" concentrations. —■— pos.control (no 2-5A added) , —◇— 1 nM 2-5A, —○— 100 nM 2-5A, —▲— 1000 nM 2-5A, —□— neg. control (no TMV RNA added).

control (Fig. 3). Laser densitometer scanning revealed an equal reduction in translation of all four major proteins.

Covalent cross-linking of [^{32}P]pCp-labeled 2-5A to proteins in cell extracts

The chemical crosslinking of [^{32}P]pCp-labeled 2-5A "cores" to proteins in cell extracts and the subsequent separation of labeled products by electrophoresis in SDS gels revealed an approximately 70 kD 2-5A-binding protein in potato leaf extracts (FIG. 4, line 5) and an 80 kD protein in mouse L-cell extracts (FIG. 4, line 3). A weak 70 kD band was also observed in WGE (data not shown). In mouse L-cell extracts, an additional minor crosslinked protein of approximately 40 kD was also found. The specificity of the binding reaction in potato leaf extracts (FIG. 4, line 4) and mouse L-cell extract (FIG. 4, line 2) was shown in competition assays where labeled 2-5A was completely displaced by unlabeled 2-5A. When the chemical crosslinking experiment was repeated with potato leaf extracts using A_3[^{32}P]pCp, a more potent protein synthesis inhibitor in plant cells, essentially the same results were obtained (data not shown).

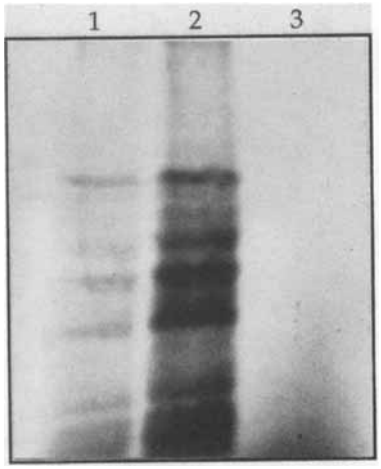

FIG. 3. The inhibition of BMV RNA translation in WGE by 2-5A tetramer "core". Translation products were labeled with [^{35}S]Met, separated by electrophoresis in 12% SDS-PAGE, dried, and autoradiographed. 1 - BMV RNA translation with 1 μM of 2-5A$_4$ "core", 2 - BMV RNA translation without 2-5A, 3 - no added RNA.

When ^{32}P-labeled 3-5A tetramer "core" was chemically crosslinked to potato leaf extract, no proteins were detected (FIG. 4, line 6), even after the very long exposure times.

Surprisingly, when mouse spleen extract was used for chemical crosslinking under the same conditions, no 2-5A-labeled proteins were detected (data not shown). Using an independent method of UV crosslinking, we found 80 kD and 40 kD 2-5A tetramer-binding proteins in mouse spleen extract (FIG. 4, line 1), which were efficiently displaced by nonlabeled 2-5A tetramer (data not shown). In potato leaf extracts, UV crosslinking did not produce any labeled proteins. The results of the UV crosslinkings of ^{32}P-labeled 3-5A tetramer "cores" with mouse spleen and potato leaf extracts were also negative (data not shown).

TMV RNA degradation in cell-free systems

The time dependence of RNA degradation in the presence or absence of 2-5A trimer triphosphate or trimer "core" in WGE was studied. Addition of trimer "core" led to a rapid degradation of TMV RNA in WGE, which was

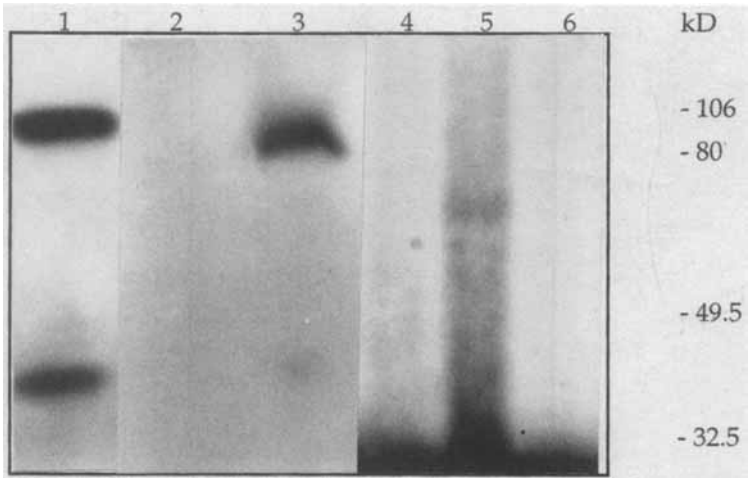

FIG. 4. Covalent binding of [^{32}P]pCp-labeled 2-5A (lines 1--5) and 3-5A (line 6) tetramer "cores" to proteins in cell extracts, using the periodate oxidation method and UV-crosslinking. 100 nM of [^{32}P]pCp-labeled 2-5A was used per reaction. Electrophoresis in 12% SDS-polyacrylamide gel. 1 - UV-crosslinking of mouse spleen extract, 2 - chemical crosslinking of mouse L-cell extract in the presence of unlabeled 2-5A, 3 - chemical crosslinking of mouse L-cell extract, 4 - chemical crosslinking of potato leaf extract in the presence of unlabeled 2-5A, 5 - chemical crosslinking of potato leaf extract, 6 - chemical crosslinking of potato leaf extract with 3-5A[^{32}P]pCp . The apparent molecular masses of protein standards are indicated in kD.

not observed with control RNA (FIG. 5). As expected, in RRL the addition of 2-5A trimer triphosphate caused rapid degradation of TMV RNA. $pppA_3$ treated TMV RNA in WGE was almost as stable as the control sample without 2-5A (FIG. 5). 2-5A "core"-dependent TMV RNA degradation indicates the presence of a 2-5A "core" activated ribonuclease in plant extracts. The opposite effects of different 2-5A forms on RNA degradation rates in WGE and RRL indicate also that the protein synthesis inhibition was not caused by ribonuclease contamination in 2-5A samples.

Inhibition of protein synthesis in protoplasts

Nonphosphorylated derivatives of 2-5A, in contrast to phosphorylated, are able to penetrate through the mammalian cell membrane.[31,32] Our results

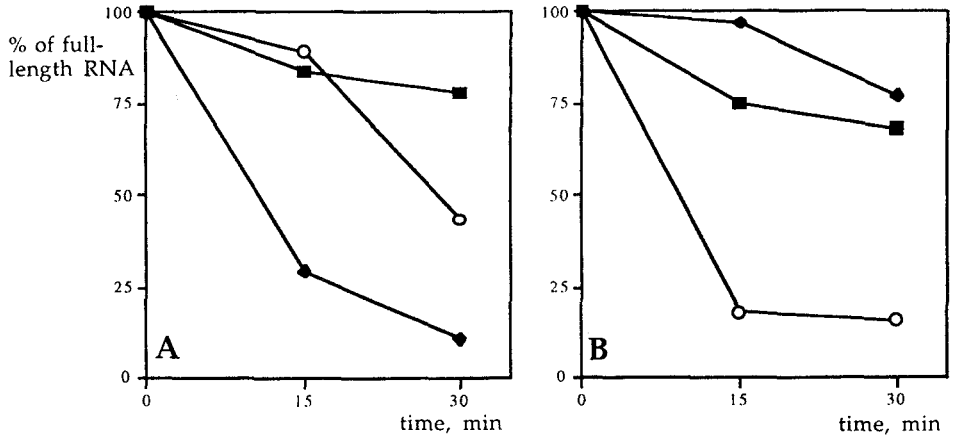

FIG. 5. The effect of 1 μm concentration of 2-5A trimer triphosphate and trimer "core" on TMV RNA degradation in WGE (A) and in RRL (B). RNA was electrophoresed on 1% agarose/formamide gels, blotted to nylon filter and hybridized with ^{32}P-labeled TMV cDNA. The autoradiographs were scanned with a laser densitometer. —■— pos. control (no 2-5A added), —◆— A_3, —○— $pppA_3$

show that this may also occur in plant protoplasts. The addition of 2-5A trimer "cores" to *N. tabacum* SR1 protoplasts (final concentration 1 μm) reduced the rate of protein synthesis by up to two-fold (FIG. 6). The control experiment demonstrated that total cellular radioactivity in the presence or absence of 2-5A remained the same when *de novo* protein synthesis was blocked by cycloheximide and the protoplasts were exposed to radioactive amino acids (data not shown). This indicates that lower incorporation of labeled amino acids in the presence of 2-5A was not due to the inhibition of cellular amino acid uptake but rather to protein synthesis inhibition in both *in vitro* and *in vivo* in plant systems.

DISCUSSION

In contrast to mammalian cell-free systems, 2-5A trimer and tetramer "cores" were the most potent inhibitors of the *in vitro* translation in the plant cell-free system. The inhibitory effect of phosphorylated 2-5A derivatives was significantly lower. Therefore, the inhibitory effect of "cores"

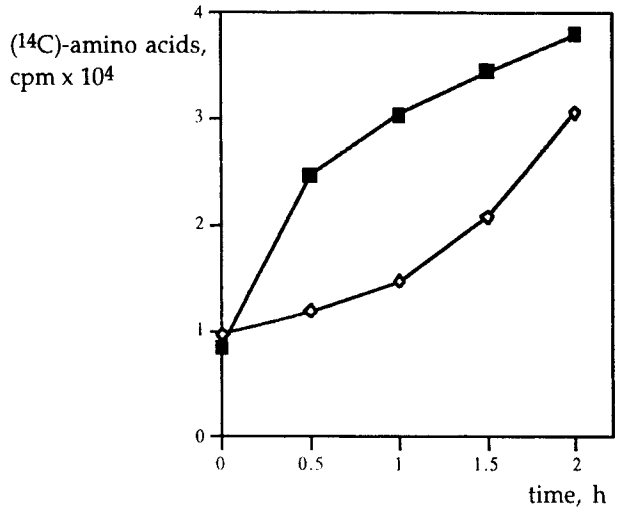

FIG. 6. The effect of 2-5A trimer "cores" on *in vivo* protein synthesis in *N.tabacum* SR1 protoplasts. —■— without 2-5A, —◇— with 1μM 2-5A_3

due contamination by phosphorylated 2-5A forms is ruled out. The purity of "cores" was shown also by HPLC analysis. The contamination of "core" preparations with enzymes such as phosphatases is also very unlike, as these were prepared using direct chemical synthesis. The concentration of 2-5A needed for efficient inhibition of viral RNA translation in WGE was about 1 μM (FIG. 2 A and B), which is about two to three orders of magnitude higher than that needed for the inhibitory effect of di- or triphosphorylated 2-5A trimers and tetramers in mammalian systems.[33,34] Although relatively high concentrations of nonphosphorylated 2-5A are needed for the *in vitro* inhibition of TMV and BMV RNA translation, this inhibition is 2-5A-specific since 3-5A, ATP, AMP, and adenosine nucleotides have no effect on translation in WGE (TABLE 1). The inhibition of protein synthesis by contaminating salts or ribonucleases is ruled out since the inhibition was specific to certain 2-5A forms. "Core" forms inhibited protein synthesis in WGE, but were inactive in the mammalian cell-free system. Phosphorylated forms had an opposite effects in these two systems. In addition, we have shown that the traces of salts present in the HPLC-purified 2-5A samples do not inhibit *in vitro* protein synthesis.[35] Micromolar concentrations of 2-5A

trimer "cores" also inhibited protein synthesis *in vivo* in protoplasts of *N. tabacum* (FIG. 6). Our finding that dephosphorylated 2-5A molecules were the best inhibitors of protein synthesis in plant systems agree with the results of Devash *et al.*[2,3]

TMV RNA translation inhibition by 2-5A trimer and tetramer "cores" is accompanied by TMV RNA degradation. An activation of a putative plant 2-5A-dependent ribonuclease may occur. This degradation is not TMV-specific, since the levels of translation products of all four BMV RNAs were diminished in the presence of 2-5A tetramer "core".

We demonstrated the existence of an approximately 70 kD 2-5A-binding protein in potato leaf extracts (FIG. 4). In plant cells, the 2-5A-binding protein had a lower molecular weight than in mammalian cells - about 70 kD versus 80 kD in mouse L-cell and mouse spleen extracts. In mammalian extracts we observed a smaller 40 kD 2-5A-binding protein, which was reported by Bisbal *et al.*[36] We were able to detect plant 2-5A-binding protein by the periodate oxydation method, but not by UV crosslinking.

Three lines of evidence support the existance of a 2-5A-activated ribonuclease in plants: i) micromolar concentrations of 2-5A trimer and tetramer "cores" inhibit protein synthesis in wheat germ extract and *N.tabacum* protoplasts; ii) the addition of 2-5A "cores" induces the degradation of TMV RNA in wheat germ extract; iii) 70 kD protein is specifically crosslinked to [^{32}P]2-5A in potato leaf extracts.

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REFERENCES

1. Lengyel, P. (1982) *Annu. Rev. Biochem.*, 251-282.
2. Devash, Y., Biggs, S., & Sela, I. (1982) *Science 216*, 1415-1416.

3. Devash, Y., Gera, A., Willis, D. H., Reichman, M., Pfleiderer, W., Charubala, R., Sela, I., & Suhadolnik, R. J. (1984) *J. Biol. Chem. 259*, 3482-3486.
4. Edelbaum, O., Ilan, N., Grafi, G., Sher, N., Stram, Y., Novick, D., Tal, N., Sela, I., & Rubinstein, M. (1990) *Proc. Natl. Acad. Sci. USA 87*, 588-592.
5. Edelbaum, O., Sher, N., Rubinstein, M., Novick, D., Tal, N., Moyer, M., Ward, E., Ryals, J., & Sela, I. (1991) *Plant Mol. Biol. 17*, 171-173.
6. Reichman, M., Devash, Y., Suhadolnik, R. J., & Sela, I. (1983) *Virology 128*, 240-244.
7. Devash, Y., Reichman, M., Sela, I., Reichenbach, N. L., & Suhadolnik, R. J. (1985) *Biochemistry 24*, 593-599.
8. Kulaeva, O. N., Fedina, A. B., Burkhanova, E. A., Karavaiko, N. N., Karpeisky, M. Ya., Kaplan, I. B., Taliansky, M. E., & Atabekov, J. G. (1992)*Plant Mol. Biol. 20*, 383-393.
9. Huisman, M. J., Broxterman, H. J. G., Schellekens, H., & van Vloten-Doting, L. (1985) *Virology 143*, 622-625.
10. Loesch-Fries, L. S., Halk, E. L., Nelson, S. E., & Krahn, K. J. (1985) *Virology 143*, 626-629.
11. de Zoeten, G. A., Penswick, J. R., Horisberger, M. A., Ahl, P., Schultze, M., & Hohn, T. (1989) *Virology 172*, 213-222.
12. Sela, I., Grafi, G., Sher, N., Edelbaum, O., Yagev, H., & Gerassi, E. (1987) In *Plant resistance to viruses*, Ciba Foundation symposium 133, (Evered, D., & Harnett, S., Eds.) pp. 109-119, John Wiley & Sons, Chichester.
13. Truve, E., Aaspõllu, A., Honkanen, J., Puska, R., Mehto, M., Hassi, A., Teeri, T. H., Kelve, M., Seppänen, P., & Saarma, M. (1993) *Bio/Technology 11*, 1048-1052.
14. Truve, E., Kelve, M., Aaspõllu, A., Kuusksalu, A., Seppänen, P., & Saarma, M. (1994) *Arch. Virol. Suppl. 9*, 41-50.
15. Cayley, P. J., White, R. F., Antoniw, J. F., Walesby, N. J., & Kerr, I. M. (1982) *Biochem. Biophys. Res. Comm. 108*, 1243-1250.
16. Ehara, Y., Nakamura, S., Yoshikawa, M., Shirisawa, N., & Taira, H. (1994)*Tohoku J. Agricult. Res. 44*, 1-6.
17. Nakamura, S., Yoshikawa, M., Taira, H., & Ehara, Y. (1994) *Ann. Phytopath. Soc. Japan 60*, 691-693.

18. Kvasyuk, E. I., Kulak, T. I., Khripach, N. B., Mikhailopulo, I. A., Uhlmann, E., Charubala, R., & Pfleiderer, W. (1987) *Synthesis* , No. 6, 535-541.
19. Kvasyuk, E. I., Kulak, T. I., Kalinitchenko, E. N., Podkopaeva, T. L., Mikhailopulo, I. A., & Pfleiderer, W. (1985) *Bioorg, Khim. 11*, 1227-1238 (in Russian).
20. Fraenkel-Conrat, H. (1957) *Virology 4*, 1-4.
21. Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
22. Knight, M., Wreschner, D. H., Silverman, R. H., & Kerr, I. M. (1981) *Meth. Enz. 79*, 216-227.
23. Brown, R. E., Cayley, P. J., & Kerr, I. M. (1981) *Meth. Enz. 79*, 208-216.
24. Wreschner, D. H., Silverman, R. H., Jones, J. C., Gilbert, C. S., & Kerr, I. M. (1982) *Eur. J. Biochem. 124*, 261-268.
25. Nolan-Sorden, N. L., Lesiak, K., Bayard, B., Torrence, P. F., & Silverman, R. H. (1990) *Anal. Biochem. 184*, 298-304.
26. Toots, U. E., Kelve, M. B., & Saarma, M. Yu. (1988) *Mol. Biol. (USSR) 22*, 1473-1481 (in Russian).
27. Feinberg, A. P., & Vogelstein, B. (1983) *Anal. Biochem. 132*, 6-13.
28. Murashige, T., & Skoog, F. (1962) *Phys. Plant. 15*, 473-497.
29. Honkanen, J., Ryöppy, P., & Tigerstedt, P. M. A. (1988) In *Biotechnology in tropical crop improvement: proceedings of the international biotechnology workshop*. pp. 81-85, ICRISAT, Patancheru, A.P.
30. Nagy, J. I., & Maliga, D. (1976) *Z. Pflanzenphysiol. 78*, 453-455.
31. Kimchi, A., Shure, H., & Revel, M. (1981) *Eur. J. Biochem. 114*, 5-10.
32. Suhadolnik, R. J., Doetsch, P. W., Devash, Y., Henderson, E. E., Charubala, R., & Pfleiderer, W. (1983) *Nucleos. Nucleot. 2*, 351-366.
33. Floyd-Smith, G., Slattery, E., & Lengyel, P. (1981) *Science 212*, 1030-1032.
34. Wreschner, D. H., McCauley, J. W., Skehel, J. J., & Kerr, I. M. (1981) *Nature (London) 289*, 414-417.
35. Kuusksalu, A., Pihlak, A., Müller, W. E. G., & Kelve, M. (1995) *Eur. J. Biochem. 232*, 351-357.
36. Bisbal, C., Salehzada, T., Lebleu, B., & Bayard, B. (1989) *Eur. J. Biochem. 179*, 595-602.

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