Expression of poliovirus $2A^{pro}$ in mammalian cells: effects on translation

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Abstract **Poliovirus protease $2A^{pro}$ has been efficiently expressed in HeLa and COS cells upon transfection with vector pTM1-2A and infection with the recombinant vaccinia virus bearing the T7 RNA polymerase. The expressed poliovirus protease localizes to the cytoplasm of the transfected cells, both in the endoplasmic reticulum and in vesicles scattered in the cytoplasm. Cleavage of p220, a component of initiation factor eIF-4F, selectively occurs from 5 h post-infection in transfected cells infected with the recombinant virus. This cleavage correlates in time with the profound inhibition observed in the synthesis of vaccinia virus proteins. A similar blockade of vesicular stomatitis virus translation takes place upon $2A^{pro}$ expression. Finally, the synthesis of poliovirus protein 2C from a recombinant vaccinia virus that expresses this protein under the EMC untranslated leader region is not affected by the synthesis of $2A^{pro}$. These findings lend support to the idea that translation of capped mRNAs requires the integrity of p220, while this requirement is not observed when translation of a mRNA bearing a picornavirus leader region is assayed.**

Key words: Protease 2A; Poliovirus; p220 cleavage; Vaccinia virus

1. Introduction

Translation of picornavirus RNA originates a large polyprotein precursor that is proteolytically cleaved by the virus encoded proteases to the mature viral products [1–3]. Apart from a final cleavage of VP0 to generate VP2 and VP0 during virion assembly, $2A^{pro}$ and $3C^{pro}$ are the two poliovirus proteases responsible for all the cleavages on the polyprotein [1,3]. Poliovirus $2A^{pro}$ and $3C^{pro}$ not only cleave viral precursor substrates, cellular proteins are also recognized and degraded by these proteases [3,4]. Thus, the p220 component of initiation factor eIF-4F is cleaved internally once by $2A^{pro}$ probably inactivating this factor to recognize capped mRNAs [5,6]. On this basis, the possibility that p220 cleavage constitutes the basis of the inhibition of cellular translation was advanced [5]. However, subsequent evidence indicated that p220 cleavage and the shut-off of host protein synthesis by poliovirus may be unrelated phenomena [7–10]. Therefore, great interest exists to express the isolated $2A^{pro}$ in mammalian cells to assay directly its effects on translation. Early attempts aimed to test the effects of $2A^{pro}$ transient expression on reporter genes indicated that $2A^{pro}$ was deleterious to gene expression 115;3725}, being the transcription of the reporter gene more affected, than its translation [11]. Attempts to make recombinant vaccinia virus bearing $2A^{pro}$ were unsuccessful, because of the toxicity of this protein for the replication of vaccinia virus [12,13]. We have now efficiently expressed poliovirus $2A^{pro}$ in a transient manner by means of recombinant vaccinia virus that contains the T7 RNA polymerase. The effects of $2A^{pro}$ expression on translation in vaccinia virus and vesicular stomatitis virus-infected cells was assayed. Our results indicate that expression of $2A^{pro}$ and subsequent cleavage of p220 leads to substantial inhibition of translation in both systems.

2. Materials and methods

2.1. Cells and virus

HeLa and COS cells were grown in tissue-culture dishes (Nunc) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Newborn calf serum. The recombinant vaccinia virus bearing the T7 RNA polymerase (VT7) (kindly given by Dr. B. Moss, NIH, Bethesda, MD) and the vesicular stomatitis virus (VSV) were grown in HeLa cells in DMEM supplemented with 2% Newborn calf serum.

2.2. Plasmid constructions

The expression plasmid pTM1-2A was constructed using standard polymerase chain reaction (PCR) techniques. Oligonucleotides were designed to hybridize with the regions 3386–3403 (primer 5′ 2A.B1/E1: CCC GGG GAT ATC ATG GGATTCGGACACCAAAAC) and 3832–3815 (primer 3′ 2A.E1A: GGG CCC GAG CTC AGG CCT TAC TA TTGTTCCATGGCTTCTTC) of poliovirus type 1 cDNA cloned in vector pT7XLD. Amplified products were purified using the GeneClean kit. The DNA preparation amplified was doubly digested with *Eco*RV + *Sac*I and ligated to pTM1 vector [14] digested with *Sac*I and blunt ended *Nco*I. Recombinant vaccinia virus VTM2C was prepared essentially as described [15,16].

2.3. Transfection of DNAs with the VT7 expression system

For transfection experiments, cells were plated in 24-well dishes (Nunc) 24 h before infection with VT7 (m.o.i. 5). After 45 min of virus adsorption, a mixture of DNA (0.5 μg/well) and Lipofectin (2 μg/well) was added to cells in DMEM as described by the manufacturer (GIBCO, BRL). Cells were harvested at the times indicated in figure legends.

2.4. Protein analysis by SDS-PAGE. Immunoblot assays

Cells were labelled with 25 μCi/ml [^{35}S]methionine (1.45 Ci/mmol, Amersham) in methionine-free medium. The radiolabelled cell monolayers were dissolved in buffer and electrophoresed as indicated [16]. Samples used for immunoblot analysis of p220 were harvested in sample buffer with 1 mM PMSF at the indicated times. After boiling, the samples were applied and electrophoresed as indicated in SDS-PAGE gels in each figure legend. Proteins were transferred to a nitrocellulose membrane (trans-blot transfer medium, Bio-Rad) overnight at 200 mA in a transfer buffer (25 mM Tris-HCl, (pH 8.3), 90 mM glycine, 20% methanol). Prelabelled molecular weight markers (Bio-Rad) were used to allow cutting the nitrocellulose sheet at the desire position. Thus, the nitrocellulose with the proteins above 16 kDa were incubated with rabbit polyclonal antibodies against p220 [17], and proteins below 16 kDa were incubated with rabbit polyclonal antibodies against $2A^{pro}$ [18]. The membrane was developed by the luminol-luciferin system and exposed to X-Ray film for 10 s to 15 min depending on the signal.

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2.5. Immunomicroscopy

For immunofluorescence microscopy HeLa cells were washed three times with PBS and fixed with 20% methanol 2 min. After fixation the cells were washed with PBS. After rinsing the cells three times, primary antibody binding was visualized with goat anti-rabbit FITC or goat anti-mouse RITC diluted in PBS for 1 h (the polyclonal primary antibody was added first). After three washes in PBS the cover slips were mounted on glass slides in mowiol and viewed with a Nikon Fluophot microscope. Staining of the endoplasmic reticulum was carried out with anti-PDI (Protein disulphide isomerase) antibodies (1D3, kindly given by Dr. D. Füller, EMBL, Heidelberg).

3. Results

Initially, the poliovirus $2A^{pro}$ was cloned in vector pTM1 as described for other poliovirus proteins [16]. The expression of this protein in HeLa cells was tested by immunofluorescence. To this end, cells transfected or not with pTM1-2A and infected with recombinant vaccinia virus bearing the T7 RNA polymerase (VT7) were incubated with specific polyclonal antibodies against poliovirus $2A^{pro}$. Control cells only infected with VT7 do not show immunofluorescence when reacted with this antibody, while timmuno fluorescence is clearly apparent in cells infected and transfected with pTM1-2A (Fig. 1). More than 70% of the cells show $2A^{pro}$ expression using this assay, albeit to various extents. A small percentage (about 10%) of the cells express the protease to high levels. The intracellular distribution of the protein shows its localization in the endoplasmic reticulum and some additional vesicles located in the cytoplasm. Specific staining of the endoplasmic reticulum in these cells was achieved by means of anti-PDI antibodies (1D3) (Fig. 1). PDI is a marker of the endoplasmic reticulum [19].

The activity of poliovirus $2A^{pro}$ on p220 cleavage and vaccinia virus translation was examined at the times shown in Fig. 2. Infection of COS cells with recombinant vaccinia virus alone does not affect the integrity of p220 (Fig. 2; panel A), whereas infection plus transfection with pTM1-2A leads to substantial cleavage of p220 after 5 h p.i. and more drastically after 24 h of infection. Profound inhibition of vaccinia virus protein synthesis is observed from 5 h p.i. in cells transfected with pTM1-2A (Fig. 2C). The extent of this inhibition and the fact that there is little p220 left intact indicate that the percentage of transfected cells is very high. Therefore, this system is well suited to test the action of poliovirus $2A^{pro}$ on translation, overcoming the construction of recombinant vaccinia virus bearing $2A^{pro}$. The actual synthesis of poliovirus $2A^{pro}$ was examined by Western blotting using polyclonal antibodies against this protease. Some expression of $2A^{pro}$ is detected 7 h p.i., while at 24 h p.i. the intensity of the band corresponding

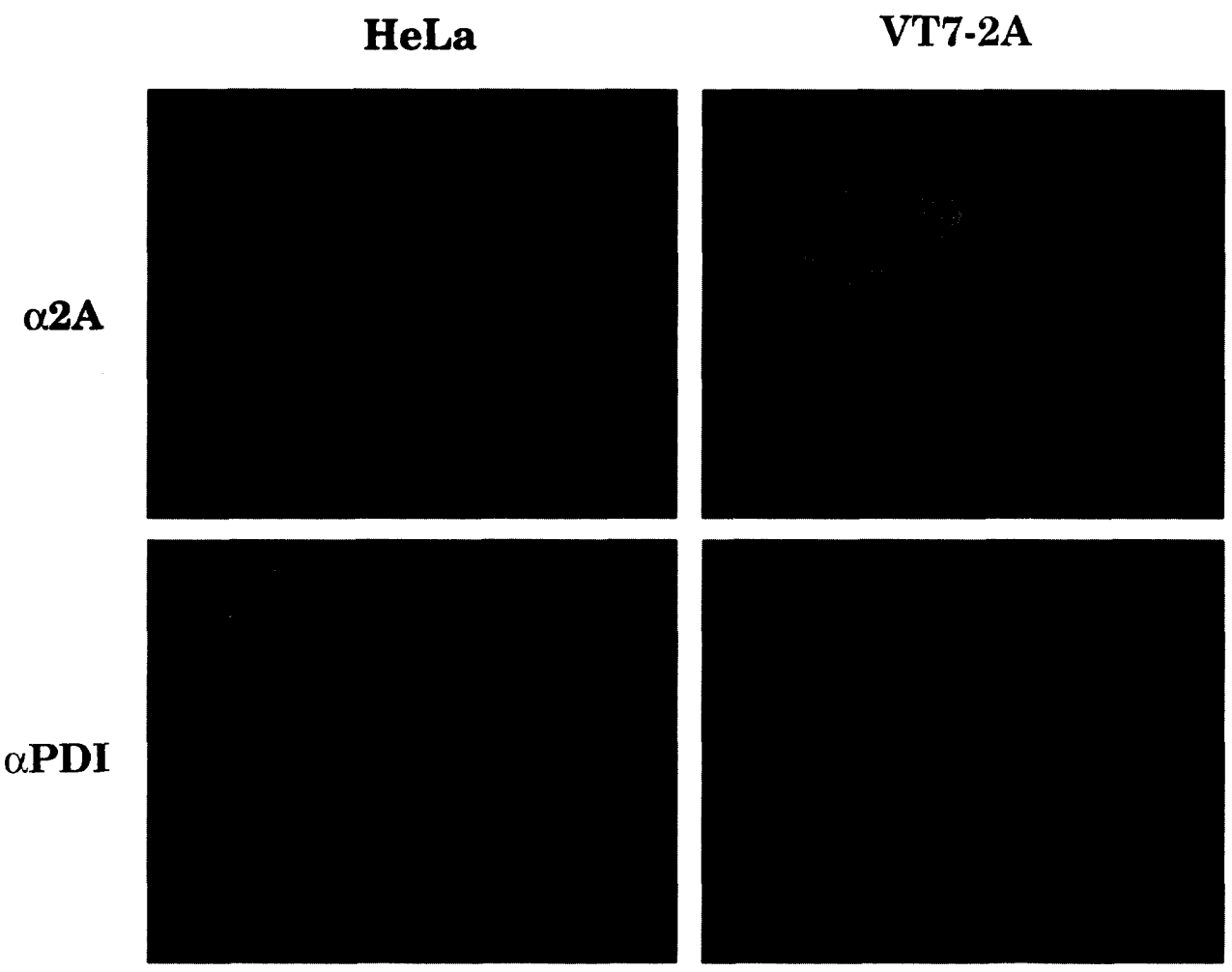

Fig. 1. Intracellular localization of poliovirus $2A^{pro}$ protein. HeLa cells were infected with VT7 (left panels) or transfected with pTM1-2A (right panels) and 8 h post-transfection immunoreacted with anti-2A antiserum (upper panels) or anti-PDI (1D3) antiserum (lower panels).

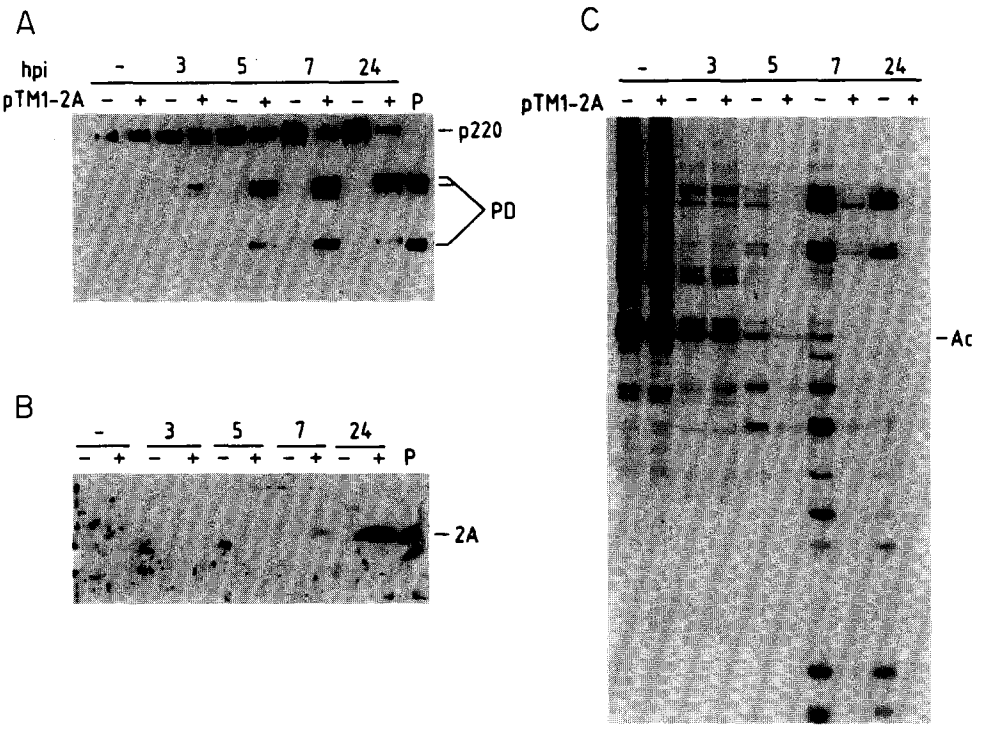

Fig. 2. Kinetics of p220 cleavage, $2A^{pro}$, expression and protein synthesis in COS cells. Cells were infected with VT7 (5 pfu/cell) and transfected with pTM1-2A plasmid. Samples were obtained at the indicated times for immunoblot analysis as described in section 2. Panel A: the blot was incubated with p220 antibodies. Panel B: the blot was incubated with 2A antibody. Panel C: proteins were labelled with [^{35}S]methionine at the indicated times for 1 h and analyzed by 15% SDS-PAGE. P, poliovirus-infected COS cells; PD, products of p220 degradation; Ac, actin.

to $2A^{pro}$ is similar to the amount of $2A^{pro}$ present in poliovirus-infected cells (Fig. 2B). Despite the low amounts of $2A^{pro}$ observed at 5 and 7 h p.i. its effects on p220 cleavage are clearly apparent (Fig. 2A).

Many of the initial effects of poliovirus on the translation of capped mRNAs, assayed VSV mRNAs as a model for cap-dependent translation [20,21]. Therefore, we decided to assay the action of poliovirus $2A^{pro}$ on the translation of VSV mRNAs. For this purpose, COS cells infected with recombinant vaccinia virus were superinfected with VSV previously transfected or not with pTM1-2A. When vaccinia virus-infected COS cells are superinfected with VSV, the proteins encoded by VSV are clearly apparent, although their level of expression is reduced as compared to cells infected with VSV alone (Fig. 3A). Notably, the synthesis of VSV proteins is very much reduced in COS cells previously transfected with poliovirus $2A^{pro}$ (Fig. 3A). These findings indicate that $2A^{pro}$ is not only deleterious for vaccinia virus translation, but also the synthesis of VSV proteins is greatly compromised by the expression of the poliovirus protease.

Finally, the action of poliovirus $2A^{pro}$ on gene expression by a recombinant vaccinia virus that synthesizes poliovirus protein 2C under the control of the EMC 5'UTR was assayed. Poliovirus 2C is one of the major proteins synthesized by this recombinant vaccinia virus at late times of infection (Fig. 3B). Gene expression in doubly infected cells with VT7 and vTM2C transfected with pTM1-2A is profoundly depressed, in agreement with the idea that protease $2A^{pro}$ blocks vaccinia virus translation. Notably, the synthesis of protein 2C by the recombinant vaccinia virus is not affected by $2A^{pro}$. These findings indicate that the translation of a mRNA incorporating the EMC 5'UTR is much less affected by poliovirus $2A^{pro}$ than the translation of the rest of vaccinia virus mRNAs. These in vivo observations lend support to the idea that internal initiation of this picornavirus UTR does not require an intact p220.

4. Discussion

The finding that $2A^{pro}$ cleaves p220 provides an useful tool to assay the requirement of this polypeptide in translation [22]. Two major approaches have been followed. The first one involves the addition of picornavirus proteases to cell lysates in order to deplete for p220. Upon cleavage of p220, the translation of capped or uncapped mRNAs is assayed [23]. The other approach involves the expression of $2A^{pro}$ in intact cells to assay for its effects on gene expression [11,24]. Addition of several picornavirus $2A^{pro}$ to cell-free systems results in efficient cleavage of p220 [25–27]. However, the effects of this cleavage on the translation of capped mRNAs is still conflicting [28,29]. Initial studies found substantial (but not total) inhibition of globin mRNA translation [30], but more recent experiments show only marginal, about 30% in some instances [29], or none inhibition [28]. Interestingly, translation of naturally or even artificially uncapped mRNAs is sometimes stimulated by the presence of the picornavirus protease in the cell-free system [28,29]. Curiously, no studies with poliovirus $2A^{pro}$ have yet been performed using cell free lysates. It remains still unclear if the enhanced translation by picornavirus protease on uncapped mRNAs is due to the actual presence of the protease, or to the cleavage of p220 and consequent enhanced activity of the cleaved prod-

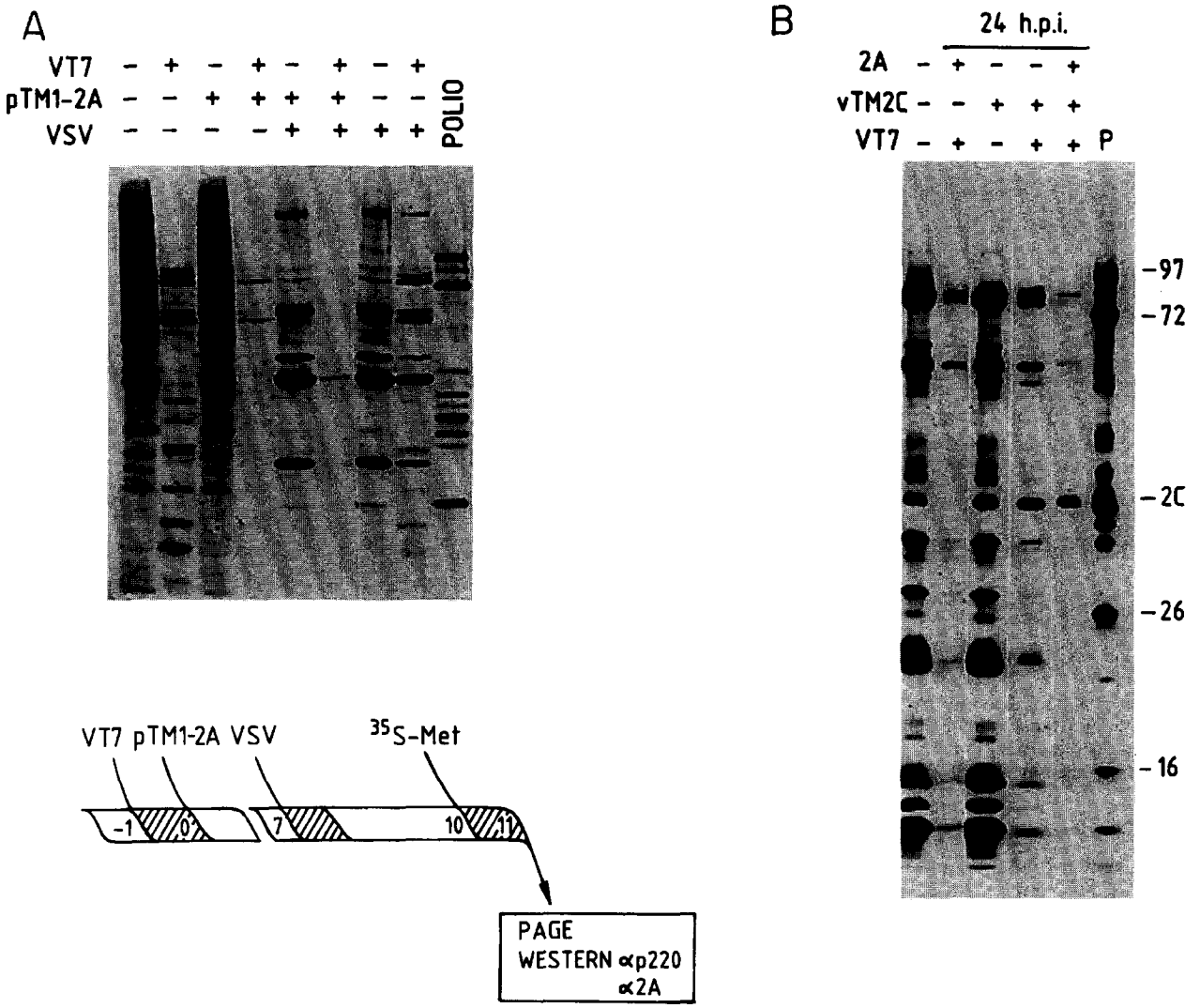

Fig. 3. Panel A: effects of $2A^{pro}$ expression on protein synthesis in cells co-infected with VSV and VT7 expressing $2A^{pro}$. COS cells grown in 24-well dishes were infected with VT7 (m.o.i. 5), and after virus adsorption transfected with pTM1-2A. At 7 h.p. transfection cells were superinfected with VSV (m.o.i. 50). Proteins were labelled with [^{35}S]methionine at 10 h p.t. for 1 h. Panel B: effects of $2A^{pro}$ expression on poliovirus 2C synthesis from recombinant vaccinia virus. COS cells coinfected with VT7 (m.o.i. 5) and VTM2C (m.o.i. 5) were transfected with pTM1-2A and 24 h p.i. proteins were labelled for 1 h at the indicated time. All the samples were analyzed by 15% SDS-PAGE.

ucts. The stimulation of translation of uncapped mRNAs was initially observed in whole cells using the poliovirus $2A^{pro}$ [31]. Therefore, it seems possible that poliovirus $2A^{pro}$ is a multifunctional enzyme. This protease cleaves p220 and blocks gene expression of reporter genes [11,24] and potently inhibits the synthesis of some viral protein (the present work). In addition $2A^{pro}$ stimulates translation of its own mRNAs [31], resembling in this respect the activity of other viral proteins as HIV rev [32,33], influenza NS1 [34].

The role that $2A^{pro}$ plays in the shut-off of host translation induced by poliovirus is still much debated [7–10]. The possibility that apart from p220 cleavage, there is a second event responsible for this effect has been speculated [35,36]. However, the present work shows that the individual expression of poliovirus $2A^{pro}$ restricts the translation of both vaccinia virus and VSV protein synthesis, leaving unnecessary the existence of a second event. Our present results together with previous findings on the inhibition of reporter genes by $2A^{pro}$ clearly point to the toxic effects of this protease for gene expression. Noteworthy, in all these studies translation of newly made mRNAs was tested, while the poliovirus-induced inhibition of cellular translation occurs on the translation of mRNAs already engaged in translation. Previous findings from several laboratories indicate that p220 cleavage can be separated from the inhibition of protein synthesis in poliovirus-infected HeLa cells [7–10]. The possibility that $2A^{pro}$ blocks expression of newly made mRNAs, having less or no effect on ongoing translation is open. Experiments are now in progress in our laboratory to test this possibility.

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References

[1] Krausslich, H.G. and Wimmer, E. (1988) Annu. Rev. Biochem. 57, 701–754.
[2] Harber, J. and Wimmer, E. (1993) in: Regulation of Gene Expression in Animal Viruses (Carrasco, L., Sonenberg, N. and Wimmer, E., Eds.), pp. 189–224, Plenum Press, London.
[3] Carrasco, L. (1994) Pharmacol. Ther. 64, 215–290.
[4] Urzáinqui, A. and Carrasco, L. (1989) J. Virol. 63, 4729–4735.
[5] Etchison, D., Milburn, S.C., Edery, I., Sonenberg, N. and Hershey, J.W. (1982) J. Biol. Chem. 257, 14806–14810.
[6] Sonenberg, N. (1990) Curr. Top. Microbiol. Immunol. 161, 23–47.
[7] Bonneau, A.M. and Sonenberg, N. (1987) J. Virol. 61, 986–991.
[8] Perez, L. and Carrasco, L. (1992) Virology 189, 178–186.
[9] Lloyd, R.E. and Bovee, M. (1993) Virology 194, 200–209.
[10] Irurzun, A., Sanchez-Palomino, S., Novoa, I. and Carrasco, L. (1995) J. Virol.
[11] Davies, M.V., Pelletier, J., Meerovitch, K., Sonenberg, N. and Kaufman, R.J. (1991) J. Biol. Chem. 266, 14714–14720.
[12] Jewell, J.E., Ball, L.A. and Rueckert, R. (1990) J. Virol. 64, 1388–1393.
[13] Turner, P.C., Young, D.C., Flanegan, J.B. and Moyer, R.W. (1989) Virology 173, 509–521.
[14] Elroy-Stein, O. and Moss, B. (1990) Proc. Natl. Acad. Sci. USA 87, 6743–6747.
[15] Cremer, K.J., Mackett, M., Wohlenberg, C., Notkins, A.L. and Moss, B. (1985) Science 228, 737–740.
[16] Aldabe, R. and Carrasco, L. (1995) Biochem. Biophys. Res. Commun. 206, 64–76.
[17] Feduchi, E., Aldabe, R., Novoa, I. and Carrasco, L. (1994) J. Gen. Virol.
[18] Martínez-Abarca, F., Alonso, M.A. and Carrasco, L. (1993) J. Gen. Virol. 74, 2645–2652.
[19] Tooze, J., Kern, H.F., Fuller, S.D. and Howell, K.E. (1995) J. Cell. Biol. 109, 35–50.
[20] Brown, D., Jones, C.L., Brown, B.A. and Ehrenfeld, E. (1982) Virology 123, 60–68.
[21] Ehrenfeld, E. and Lund, H. (1977) Virology 80, 297–308.
[22] Krausslich, H.G., Nicklin, M.J., Toyoda, H., Etchison, D. and Wimmer, E. (1987) J. Virol. 61, 2711–2718.
[23] Liebig, H.-D., Ziegler, E., Yan, R., Hartmuth, K., Klump, H., Kowalski, H., Blaas, D., Sommergruber, W., Frasel, L., Lamphear, B., Rhoads, R., Kuechler, E. and Skern, T. (1993) Biochemistry 32, 7581–7588.
[24] Sun, X.H. and Baltimore, D. (1989) Proc. Natl. Acad. Sci. USA 86, 2143–2146.
[25] Sommergruber, W., Ahorn, H., Zöphel, A., Maurer-Fogy, I., Fessl, F., Schnorrenberg, G., Liebig, H.-D., Blaas, D., Kuechler, E. and Skern, T. (1992) J. Biol. Chem. 267, 22639–22644.
[26] Sommergruber, W., Ahorn, H., Klump, H., Seipelt, J., Zoephel, A., Fessl, F., Krystek, E., Blaas, D., Kuechler, E., Liebig, H.-D. and Skern, T. (1994) Virology 198, 741–745.
[27] Kirchweger, R., Ziegler, E., Lamphear, B.J., Waters, D., Liebig, H.-D., Sommergruber, W., Sobrino, F., Hohenadl, C., Blaas, D., Rhoads, R.E. and Skern, T. (1994) J. Virol. 68, 5677–5684.
[28] Ziegler, E., Borman, A.M., Kirchweger, R., Skern, T. and Kean, K.M. (1995) J. Virol. 69, 3465–3474.
[29] Ohlmann, T., Rau, M., Morley, S.J. and Pain, V.M. (1995) Nucleic Acids Res. 23, 334–340.
[30] Hunt, T. and Ehrenfeld, E. (1971) Nature New Biol. 230, 91–94.
[31] Hambidge, S.J. and Sarnow, P. (1992) Proc. Natl. Acad. Sci. USA 89, 10272–10276.
[32] D'Agostino, D.M., Felber, B.K., Harrison, J.E. and Pavlakis, G.N. (1992) Mol. Cell. Biol. 12, 1375–1386.
[33] Malim, M.H. and Cullen, B.R. (1991) Cell 65, 241–248.
[34] Qian, X.-Y., Alonso-Caplen, F. and Krug, R.M. (1994) J. Virol. 68, 2433–2441.
[35] Wyckoff, E.E., Hershey, J.W. and Ehrenfeld, E. (1990) Proc. Natl. Acad. Sci. USA 87, 9529–9533.
[36] Wyckoff, E.E., Lloyd, R.E. and Ehrenfeld, E. (1992) J. Virol. 66, 2943–2951.