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Poliovirus $2A^{pro}$ expression inhibits growth of yeast cells

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Abstract Poliovirus encodes two proteases, $2A^{pro}$ and $3C^{pro}$ that participate in the processing of the viral polyprotein and cleave a number of host proteins. Both proteases have been cloned and expressed in an inducible manner in *Saccharomyces cerevisiae* cells. The expression of $2A^{pro}$, but not $3C^{pro}$, was highly toxic for yeast cells such that growth was arrested after 5 h of induction and cell survival sharply declined. Cellular morphology was profoundly modified by expression of poliovirus $2A^{pro}$, in such a way that electron dense granules and autophagosomic bodies arise in the cytoplasm. Experiments aimed at defining the yeast function affected by $2A^{pro}$ suggested that translation was not the target of protease toxicity, but showed that RNA synthesis was profoundly blocked.

Key words: Poliovirus; *Saccharomyces cerevisiae*; Poliovirus protease 2A; Toxic protein

1. Introduction

One of the best understood members of the *Picornaviridae* family of animal viruses is poliovirus [1]. It contains a 7.4 kb RNA genome that encodes a polyprotein which is proteolytically cleaved, to produce eleven mature virus proteins, by two virus-encoded proteases – $2A^{pro}$ and $3C^{pro}$ [2,3]. $2A^{pro}$ cleaves Tyr–Gly bonds, while $3C^{pro}$ carries out most of the cleavages of the polyprotein between Gln–Gly bonds. Although specific poliovirus proteins participate in cytopathogenicity of the host cell during poliovirus growth, the mechanisms involved remain poorly understood. Such modification includes an increase in phospholipid synthesis [4], membrane proliferation [5], inhibition of host translation [6,7] and modification of membrane permeability [8–10]. Since all poliovirus proteins are synthesized in equal proportions throughout the virus life cycle it is difficult to assess which viral protein(s) is responsible for modifying a given cellular process. Thus, $2A^{pro}$ and $3C^{pro}$ not only participate in virus replication but also appear to inhibit essential host functions and play a role in the virus-induced cytopathic effects [1,11]. To get further insight into the effects of poliovirus proteases on cellular functions, it is useful to analyze the toxicity of the individual expression of each viral protease on eukaryotic cells.

We decided to use the yeast *S. cerevisiae* for expression of the poliovirus non-structural proteins to assay the potential effects of individual poliovirus proteins in eukaryotic cells [12], because the ease of genetic analyses in yeast cells. This strategy had been successfully used to study the citotoxic effects of expression of human p53 [13] or the viral $pp60^{v\text{-}src}$ [14,15]. Our results indicate that poliovirus protein 2A is very toxic for *S. cerevisiae* cells and the major inhibitory effects of the protease were at the transcriptional level.

2. Materials and methods

2.1. Microbial strains

E. coli DH5 [16] was used for the construction of all expression plasmids. The *S. cerevisiae* strain used was W303-1B (Mat α, Ade-2, His-3, Leu-2, Trp-1, Ura-3), generously provided by Dr. J.P.G. Ballesta (Centro de Biología Molecular, Madrid, Spain).

2.2. Plasmid constructions

Construction of vectors was carried out by standard procedures [16]. The plasmid used was the yeast-*E. coli* shuttle vector pEMBLyex4 [17] (generously provided by E. Hernandez, University of Lleida, Spain), a 2 μ plasmid derivative. Poliovirus 2A, 2AB and 3C sequences were amplified by PCR from a cloned cDNA of poliovirus type 1 (generously provided by E. Wimmer, Stony Brook, USA), their products were digested with the appropriate restriction enzymes and cloned in pEM-BLyex4. Plasmids pEMBL.2A, pEMBL.2AB and pEMBL.3C encoded the indicated poliovirus proteins with an additional methionine at the N terminus. Constructions were confirmed by DNA sequencing following the dideoxy method [16].

2.3. Yeast media, growth, transformation and induction

Yeast cells were transformed and selected in minimal YNB plates containing 0.67% yeast nitrogen base without amino acids, and 2% of either glucose (YNB.Glu) or galactose (YNB.Gal) plus 20 mg/l of the required amino acids or bases according to the auxotrophic markers. For UAS_{GAL}-CYC promoter induction cells were grown in YNB.Lac (0.67% yeast nitrogen base without amino acids, 2% lactic acid, 0.1% glucose, 5 mM K_2HPO_4, 10 mM $(NH_4)SO_4$, adjusted to pH 6–7 with KOH), 2% galactose was added directly to YNB.Lac, after the addition of galactose this medium is referred to as YNB.LGal. Transformation of yeast by the lithium acetate procedure was performed as previously described [18].

For growth kinetics, overnight cultures grown in YNB.Lac were diluted in YNB.Lac or YNB.LGal medium, and cells were harvested at 1 to 2 h intervals at 30°C. Absorbance at 660 nm was measured to quantitate cell density. Cell viability was tested by placing aliquots of cell cultures on selective plates of YNB.Glu at different times post-induction.

2.4. Macromolecular synthesis

To measure DNA, RNA or protein synthesis, aliquots of yeast cultures were collected and incubated with 10 μCi/ml [methyl-^{3}H]thymidine (47 Ci/mmol, Amersham Corp.), [5-^{3}H]uridine (26 Ci/mmol, Amersham Corp.) or 5 μCi/ml [^{35}S]methionine (1.45 Ci/mmol, Amersham Corp.), respectively, for 15 min at 30°C. TCA precipitation was used to determine incorporation levels of radioactive precursors. Western blot analysis was carried out as described [19].

2.5. Electron microscopy

Electron microscopy, the protocol was adapted from that described by Wright et al. [20], with two modifications: yeast cells were fixed 2 h at 4°C with 2% glutaraldehide in 0.1 M cacodylate buffer, pH 6.8 and finally embedded in LR white (The London Resin Co. Ltd, Cardiff, UK).

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3. Results

3.1. Effects of $2A^{pro}$ expression on yeast growth

Fig. 1A shows that both $2A^{pro}$ clones grow well in solid medium containing glucose, whereas in galactose medium, only the yeast cells expressing $3C^{pro}$ grow at the same levels as those found in control cells bearing the vector pEMBLyex4 (results not shown). The clone that bears the 2AB insert grows to some extent in galactose medium, whereas the one containing $2A^{pro}$ sequences does not grow at all in this solid medium. These results are reinforced by measuring the kinetics of growth of these clones in liquid medium containing galactose (Fig. 1B). Yeast cultures containing the 2AB gene grow to lower rates than control cells, but growth of the clone bearing the plasmid that encodes poliovirus $2A^{pro}$ is arrested after 5 h of induction (Fig. 1B). This behaviour is not observed in cultures that express $3C^{pro}$, which grow at control levels after induction. The main conclusion from these results is that the inducible expression of poliovirus $2A^{pro}$ is not tolerated by yeast cells that stop growing a few hours after induction.

3.2. Expression of poliovirus proteases in S. cerevisiae

The ability of clones to induce the synthesis of $2A^{pro}$, 2AB or 3C in media with or without galactose was assayed, extracts were obtained after 6 and 24 h post-induction and the presence of these proteins was tested by immunoblot analysis using antiserum against $2A^{pro}$ or $3C^{pro}$. Fig. 1C shows that $2A^{pro}$ is detected in yeast cells after 6 h post-induction and the protein made has the same apparent MW as the $2A^{pro}$ synthesized in poliovirus- infected cells. After 24 h of induction in galactose medium $2A^{pro}$ is barely detected, perhaps as a reflection of the cellular degenration that occurs at this time (see below). The levels of poliovirus 2AB detected after 24 h of induction are not as high as with $2A^{pro}$ after 6 h (Fig. 1C). The reduced effects of 2AB expression on yeast growth could be due to this lower expression or to the lower protease activity of 2AB as compared to $2A^{pro}$. At present these are no sufficient data to decide between these two possibilities. It must be noted, that 2AB is not usually found in poliovirus-infected cells, since the precursor 2ABC is rapidly cleaved to 2A and 2BC and the latter is then proteolitically degraded to 2B and 2C [2].

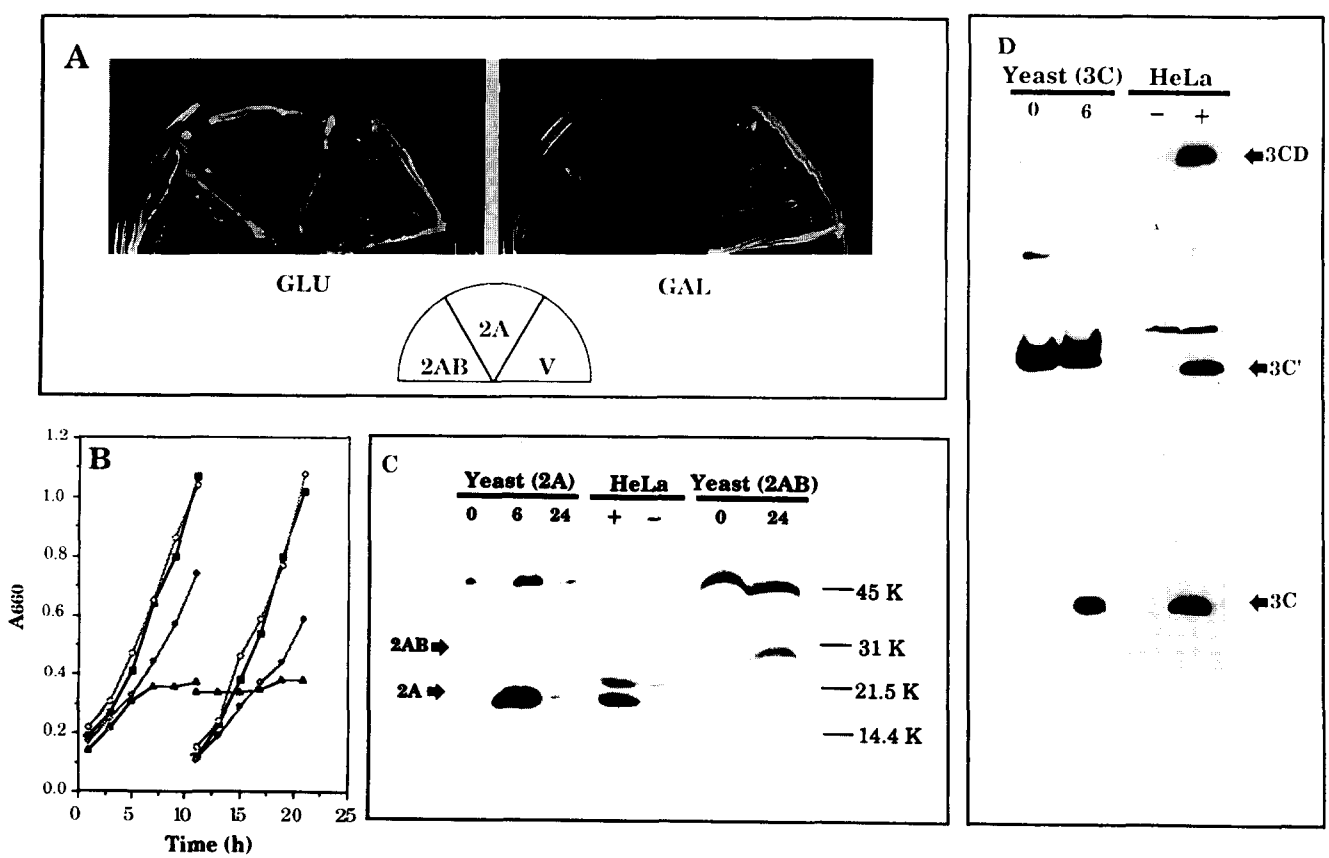

Fig. 1. Growth of yeast cells that express the poliovirus proteases. (Panel A) Yeast cells that produce different poliovirus proteins were streaked on YNB. Glu (left plate) or YNB. Gal (right plate): yeast cells transformed with the plasmids pEMBLyex4 (V), pEMBL.2A (2A) and pEMBL.2AB (2AB). (Panel B) Growth of yeast cells expressing 2A (▲), 2AB (◇) or 3C (■) poliovirus proteases induced in YNBLGal medium. Yeast cells transformed with plasmid pEMBLyex4 (○) were used as control. Two series of cultures with different dilutions were induced to prevent culture saturation and to follow growth during 20 h post-induction. (Panel C) Immunoblot analysis of 2A and 2AB proteins. (Panel D) Immunoblot analysis of 3C protein. Yeast cells containing the expression plasmid coding for the indicated poliovirus protein, were grown in YNB.Lac (–) or YNB.LGal (+) medium. After the times post-induction indicated (in hours), crude extracts were obtained as described in section 2, separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoreacted with the corresponding rabbit antisera. HeLa cells (–) or poliovirus-infected HeLa cell (+) extracts were used as controls. The position of recombinant poliovirus proteins is indicated by arrows.

Poliovirus protease $3C^{pro}$ is generated by autocleavage of the precursor 3CD to render $3C^{pro}$ and 3D. Alternative cleavage of 3CD by protease $2A^{pro}$ generates 3C′ and 3D′ [2,3]. Thus, immunoblot analyses of the proteins present in poliovirus-infected HeLa cells with anti-$3C^{pro}$ antiserum visualizes three proteins: 3CD, 3C′ and $3C^{pro}$ (Fig. 1D). Induction with galactose of yeast cells bearing plasmid pEMBL.3C originates a clear protein band that migrates as genuine $3C^{pro}$ made in the infected cells (Fig. 1D).

3.3. Effect of $2A^{pro}$ expression on yeast intracellular morphology

Examination of yeast cultures that express $2A^{pro}$ by Nomarski microscopy, after 6–9 h of induction, shows atypical yeast cells much bigger than wild-type cells and after 10 h more, these cells show a clear vacuolization (results not shown). To get further details of the morphology of cells expressing $2A^{pro}$ for different time periods, they were analyzed by transmission electron microscopy. Yeast cells bearing pEMBL.2A growing in lactate show the same ultrastructure as control cells bearing pEMBLyex4; i.e. a single and distinct nucleus and usually a single vacuole (Fig. 2A). Eight hours postinduction these cells show a different appearance, the cytoplasm becomes more electron-dense (Fig. 2); most of cells do not have a distinct nucleus, whereas spherical, electron-dense bodies accumulate, as described for autolytic processes [21]. These bodies may correspond to lipid globules; the appearance of autophagosomic bodies in the vacuoles is also frequent, as occurs under nutrient-deficient conditions [22]. Mitochondria usually retain a 'normal' appearance for a longer period, as compared to other organelles. In the last stages, after 24 h induction, most cells are filled with disorganized membranes (Fig. 2). This extensive desintegration of cytoplasmic organelles is equivalent to that described during autolysis of other fungi with micelial cells, but in yeast cells few ultrastructural studies about autolysis have been reported [23].

3.4. Effect of $2A^{pro}$ expression on yeast macromolecular synthesis and cell viability

Several cellular proteins are cleaved in poliovirus-infected cells [24], but it is not known how many of these proteins are substrates for $2A^{pro}$. The only documented case of cellular protein cleavage by poliovirus $2A^{pro}$ is on p220 [25], a component of initiation factor eIF4F, a protein involved in translation. It is unclear if p220 cleavage is responsible for the poliovirus-induced host-cell shut-off of protein synthesis or if another event is responsible for this phenomenon [6,7,26,27]. Thus, it was of interest to analyze the effects of $2A^{pro}$ expression on *S. cerevisiae* macromolecular synthesis, since the protein synthesis machinery of this organism is similar to that of mammalian cells [28] and some of the factors involved in translation in these systems are interchangeable [29].

Measurement of DNA, RNA and protein synthesis for 11 h after $2A^{pro}$ induction indicates that DNA synthesis does not increase after induction, a result that probably reflects the inhi-

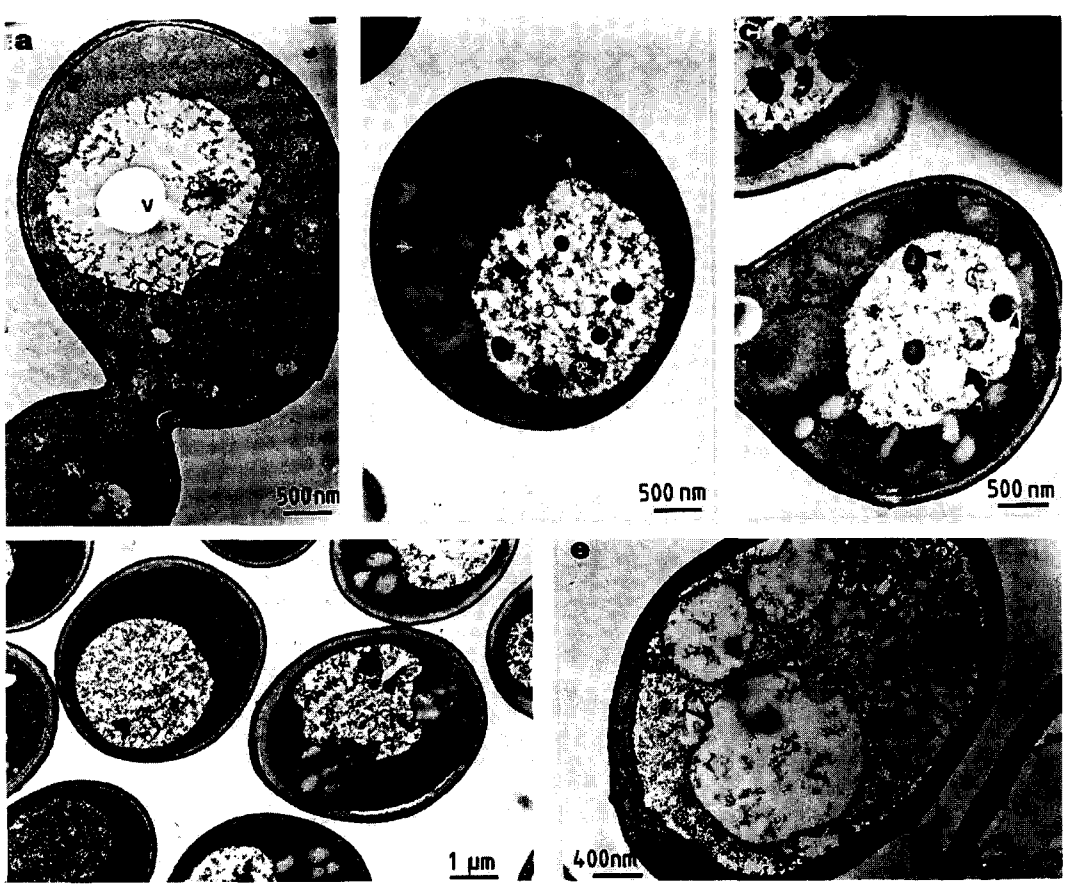

Fig. 2. Thin section electron microscopy of cells expressing $2A^{pro}$. Cells bearing pEMBL.2A at zero (A), 8 h post-induction (B, C and D) or 24 h post-induction (E) were processed for electron microscopy, and thin sections were prepared and photographed. N, nucleus; V, vacuole; M, mitochondria. White arrows: electron-dense granules. Black arrows: autophagosomic bodies.

Table 1
Macromolecular synthesis and viability in yeast cells expressing $2A^{pro}$

Yeast cells bearing plasmid/t.p.i.	A_{660} (%)	DNA synthesis (%)	RNA synthesis (%)	Protein synthesis (%)	Survival (%)
pEMBLyex4/0 h	14.1	10.7	20.0	42.5	106
pEMBLyex4/5 h	32.9	35.6	46.7	89.5	109
pEMBLyex4/11 h	**100.0**	**100.0**	**100.0**	**100.0**	**100**
pEMBL.2A/0 h	15.6	15.0	16.1	53.5	101
pEMBL.2A/3 h	26.1	22.5	16.4	48.6	5
pEMBL.2A/5 h	31.6	22.5	3.9	39.3	1.5
pEMBL.2A/7 h	38.3	20.2	2.5	36.8	–
pEMBL.2A/9 h	40.4	21.3	2.1	34.3	0.15
pEMBL.2A/11 h	40.7	19.0	1.4	32.6	0.04

To quantify cell density, the absorbance at 660 nm was measured (A_{660}). Cell viability, was tested by placing aliquots of cell cultures on selective plates of YNB.Glu at different times post-induction (t.p.i.) to repress 2A expression. The number of the initial cells was calculated from A_{660} data and the number of viable cells was obtained by counting colonies after 3 days of incubation at 30°C. Macromolecular synthesis in yeast cells bearing pEMBLyex4 or pEMBL.2A was estimated at different hours after placing the cells in YNB.LGal medium. To measure DNA, RNA or protein synthesis, aliquots of yeast cultures were collected and labeled as described in section 2.

bition of cell division. In contrast, RNA synthesis is profoundly affected (Table 1). Thus, 5 h after addition of galactose, uridine incorporation is almost totally blocked despite the fact that cell density increases during the first 4 h of induction. Nevertheless, protein synthesis continues even after 11 h of incubation in galactose-containing medium (Table 1). Analysis, by PAGE, of the proteins synthesized provides no evidence for the degradation of any particular polypeptide (results not shown). However, more sophisticated analyses, including high-resolution two-dimensional gel electrophoresis, would be required to determine unequivocally whether particular proteins are degraded. Our conclusion from these results is that yeast transcription is inhibited soon after $2A^{pro}$ induction, whereas overall protein synthesis continues, suggesting that a factor involved in translation is not the target of $2A^{pro}$ in yeast cells.

Finally, we monitored cell viability after $2A^{pro}$ induction (Table 1), only 3 h after induction of $2A^{pro}$ the number of viable cells dropped by 95%, suggesting that $2A^{pro}$ cleaves one or more yeast proteins required for cell division. It should be noted that once $2A^{pro}$ has been synthesized, this protein would remain stable for some time, even if cells are returned to a medium without galactose.

4. Discussion

In this work we have analyzed the action of the poliovirus proteases on yeast cells in an inducible manner so that potential cytotoxic effects on this eukaryotic system could be detected. Apart from the advantage of using *S. cerevisiae* rather than prokaryotic systems for the synthesis of recombinant proteins, this yeast could be a suitable host for analyzing the effects of cytotoxic proteins from animal viruses in eukaryotic cells. For example, we now show that $2A^{pro}$ is toxic to yeast cells. This poliovirus protease recognizes and cleaves between Tyr–Gly bonds, when present in a given context in proteins [30]. Only three substrates for $2A^{pro}$ are known at present: two in the viral polyprotein (junctions P1–2A and 3C′–3D′) and the other in the p220 subunit of the ribosomal initiation factor eIF-4F [31,32]. The computer comparison between the cleavage site in eIF-4F human [32] and its homolog in yeast [33] shows the absence of this site in the yeast protein (results not shown). Although also other cellular proteins that appear cleaved in poliovirus-infected cells [24] are still not known.

At least two possibilities can be put forward to explain this toxicity. $2A^{pro}$ may cleave, by accident, one or several of the yeast proteins neede for transcription, with the corresponding mammalian counterparts not being targets for this protease. Alternatively, $2A^{pro}$ toxicity in yeast may reflect its action in mammalian cells. At present there are insufficient data to distinguish between these possibilities. Expression of $2A^{pro}$ is not, however, necessarily toxic for cells. Thus, *E. coli* can synthesize this protease at high levels in an active form, without any apparent harmful effects [34,35] and by analogy it may be that the action of $2A^{pro}$ in mammalian and yeast cells differs. Thus, yeast cells that express $2A^{pro}$ are blocked in RNA synthesis, whereas $2A^{pro}$ cleaves the p220 component of eIF-4F in mammalian cells [25,26]. Individual expression of $2A^{pro}$ in mammalian cells strongly interferes with the expression of reporter genes [36, 37]. However, only in one case were the different steps inhibited by $2A^{pro}$ in reporter gene expression analyzed, with indications that the major inhibitory effect of the protease was on transcription of plasmid DNA [37]. Protein synthesis and DNA replication were less affected. On the other hand, the incapacity of yeast cells bearing pEMBL.2A to grow in plates with galactose could allow the development of a genetic assay that could be used not only to screen large number of components for specific protease inhibitors but also to detect protease-deficient mutants assayed in vivo.

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References

[1] 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, New York.
[2] Krausslich, H.G. and Wimmer, E. (1988) Annu. Rev. Biochem 57, 701–754.
[3] Hellen, C.U.T. and Wimmer, E. (1992) Virology 187, 391–397.
[4] Guinea, R. and Carrasco, L. (1990) EMBO J. 9, 2011–2016.
[5] Bienz, K., Egger, D., Pfister, T. and Troxler, M. (1992) J. Virol. 66, 2740–2747.
[6] Carrasco, L. and Castrillo, J.L. (1987) in: Mechanisms of Viral

Toxicity in Animal Cells (Carrasco, L. ed.) pp. 115–146, CRC Press, Boca-Raton.
[7] Sonenberg, N. (1990) Curr. Top. Microbiol. Immunol. 161, 23–47.
[8] Carrasco, L., Otero, M.J. and Castrillo, J.L. (1989) Pharmacol. Ther. 40, 171–212.
[9] Carrasco, L., Perez, L., Irurzun, A., Lama, J., Martinez-Abarca, F., Rodrigez, P., Guinea, R., Castrillo, J.L., Sanz, M.A. and Ayala, M.J. (1993) in: Regulation of Gene Expression in Animal Viruses (Carrasco, L., Sonenberg, N. and Wimmer, E. eds.) pp. 283–305, Plenum Press, London, New York.
[10] Carrasco, L. (1995) Adv. Virus Res. 45, 61–112.
[11] Porter, A.G. (1993) J. Virol. 67, 6917–6921.
[12] Barco, A. and Carrasco, L. (1995) Gene 156, 19–25.
[13] Nigro, J.M., Sikorski, R., Reed, S.I. and Vogelstein, B. (1992) Mol. Cell. Biol. 12, 1357–1365.
[14] Brugge, J.S., Jarosik, G., Andersen, J., Queral Lustig, A., Fedor Chaiken, M. and Broach, J.R. (1987) Mol. Cell. Biol. 7, 2180–2187.
[15] Boschelli, F. (1993) Mol. Cell. Biol. 13, 5112–5121.
[16] Sambrook, J., Fritsch, E.R. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.
[17] Cesareni, G. and Murray, J.A.H. (1987) Genetic Engineering 9, 135–153.
[18] Rose, M.D., Winston, F. and Hieter, P. (1990) Methods in Yeast Genetics. A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, New York.
[19] Barco, A. and Carrasco, L. (1995) EMBO J.
[20] Wright, R., Basson, M., D'Ari, L. and Rine, J. (1988) J. Cell. Biol. 107, 101–114.
[21] Trinci, A.P.J. and Righelato, R.C. (1970) J. Gen. Microbiol. 60, 239–249.
[22] Baba, M., Takeshige, K., Baba, N. and Ohsumi, Y. (1994) J. Cell Biol. 124, 903–913.
[23] Takeo, K., Yamamura, M. and Kamihara, T. (1989) FEMS Microbiol. Lett. 61, 297–300.
[24] Urzáinqui, A. and Carrasco, L. (1989) J. Virol. 63, 4729–4735.
[25] Etchison, D., Milburn, S.C., Edery, I., Sonenberg, N. and Hershey, J.W. (1982) J. Biol. Chem. 257, 14806–14810.
[26] Perez, L. and Carrasco, L. (1992) Virology 189, 178–186.
[27] Lloyd, R.E. and Bovee, M. (1993) Virology 194, 200–209.
[28] Müller, P.P. and Trachsel, H. (1990) Eur. J. Biochem. 191, 257–261.
[29] Altmann, M., Muller, P.P., Pelletier, J., Sonenberg, N. and Trachsel, H. (1989) J. Biol. Chem. 264, 12145–12147.
[30] Hellen, C.U.T., Lee, C.-K. and Wimmer, E. (1992) J. Virol. 66, 3330–3338.
[31] Wyckoff, E.E., Lloyd, R.E. and Ehrenfeld, E. (1992) J. Virol. 66, 2943–2951.
[32] Lamphear, B.J., Yan, R., Yang, F., Waters, D., Liebig, H.-D., Klump, H., Kuechler, E., Skern, T. and Rhoads, R.E. (1993) J. Biol. Chem. 268, 19200–19203.
[33] Goyer, C., Altmann, M., Lee, H.S., Blanc, A., Deshmukh, M., Woolford, Jr., J.L., Trachsel, H. and Sonenberg, N. (1993) Mol. Cell. Biol. 13, 4860–4874.
[34] Alvey, J.C., Wyckoff, E.E., Yu, S.F., Lloyd, R. and Ehrenfeld, E. (1991) J. Virol. 65, 6077–6083.
[35] Martínez-Abarca, F., Alonso, M.A. and Carrasco, L. (1993) J. Gen. Virol. 74, 2645–2652.
[36] Sun, X.H. and Baltimore, D. (1989) Proc. Natl. Acad. Sci. USA 86, 2143–2146.
[37] Davies, M.V., Pelletier, J., Meerovitch, K., Sonenberg, N. and Kaufman, R.J. (1991) J. Biol. Chem. 266, 14714–14720.