Co-expression of human eIF-4G and poliovirus 2Apro in 
Saccharomyces cerevisiae: effects on gene expression

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The poliovirus 5’ untranslated region (5’ UTR) confers on mRNAs the capacity to be translated by internal initiation. The functionality of this RNA motif has been tested in yeast cells (Saccharomyces cerevisiae) using luciferase (luc) as a reporter gene. Although some luciferase is synthesized from luc mRNA containing the poliovirus 5’ UTR (Leader-luc mRNA), much more luciferase is synthesized in cells that express luc mRNA devoid of the poliovirus 5’ UTR. Since poliovirus 2Apro enhances the translation of Leader-luc mRNAs after eIF-4G cleavage in mammalian cells, yeast cells were produced that express luc mRNA devoid of the poliovirus 5’ UTR. Although some luciferase is synthesized from luc mRNA containing the poliovirus 5’ UTR (Leader-luc mRNA), much more luciferase is synthesized in cells that express luc mRNA devoid of the poliovirus 5’ UTR. Since poliovirus 2Apro enhances the translation of Leader-luc mRNAs after eIF-4G cleavage in mammalian cells, yeast cells were produced that synthesize three heterologous proteins, luciferase, poliovirus, 2Apro and human eIF-4G. Initially, S. cerevisiae cells constitutively expressing human eIF-4G were isolated. The human eIF-4G gene does not complement yeast cells defective in the initiation factor counterpart, p150, indicating that the human and yeast eIF-4G are not interchangeable. Expression of poliovirus 2Apro in an inducible manner does not affect p150, but led to the efficient cleavage of human eIF-4G in yeast cells. Induction of 2Apro was detrimental to luciferase synthesis either from luc mRNA or Leader-luc mRNA irrespective of the presence or absence of human eIF-4G. 2Apro blocked luciferase expression at the translational level. Finally, the effects of 16 point mutations of poliovirus 2Apro on luciferase expression and human eIF-4G cleavage were analysed. Only those 2Apro variants that generate viable polioviruses actively cleave eIF-4G in yeast.

Introduction

The 5’ structure of mRNAs plays an important role in translational control in eukaryotic cells. Most cellular mRNAs bear a cap structure at the 5’ terminus necessary for ribosome binding, a process which is mediated by an initiation factor complex known as eIF-4F (reviewed by Merrick & Hershey, 1996; Pain, 1996; Sonenberg, 1996). This complex is formed by a major subunit, the initiation factor eIF-4G, a cap-binding protein, eIF-4E, and a helicase, eIF-4A (Pain, 1996; Merrick & Hershey, 1996). The eIF-4G protein contains two independent domains (Lamphear et al., 1995): the N-terminal domain interacts with eIF-4E (Mader et al., 1995) while the C-terminal domain binds eIF-4A and eIF-3, a factor involved in the interaction with mRNA (reviewed by Hentze, 1997; Sachs et al., 1997). The translational machinery is highly conserved in evolutionarily terms amongst eukaryotic cells. Thus, mammalian eIF-4E or some subunits of eIF-2 can substitute for their yeast homologues in vivo (Altmann et al., 1989; Merrick & Hershey, 1996). Moreover, yeast eIF-3 can also replace its mammalian counterpart in an in vitro essay of the initiation of translation (Naranda et al., 1994). However, mouse eIF-4A, a component of the eIF-4F complex, does not complement yeast eIF-4A (Prat et al., 1990). In the case of eIF-4G there are some differences between the yeast and human counterparts. Yeast eIF-4G, also known as p150, is encoded by two genes, TIF4631 and TIF4632, that express two proteins, eIF-4G1 and eIF-4G2, which are 53% identical (Goyer et al., 1993). Deletion of one of the two genes does not severely affect yeast growth, while deletion of both genes is lethal for the cell (Goyer et al., 1993).

Several members of the family Picornaviridae, including poliovirus, encode a protease that cleaves eIF-4G, separating the two functional domains of this factor (Etchison et al., 1982; Liebig et al., 1993; Lamphear et al., 1995). The viral protease responsible for eIF-4G cleavage is known as L in aphthoviruses, while in entero- and rhinoviruses cleavage is accomplished by the protease 2Apro (Ryan & Flint, 1997; Ehrenfeld, 1996). Translation of picornavirus RNA is not affected by eIF-4G cleavage because those mRNAs only require the C terminus of eIF-4G bound to eIF-4A (Lamphear et al., 1995; Pestova et al., 1996; Ohlmann et al., 1996). It has not been definitively...
proven that poliovirus 2A\textsuperscript{pro} directly cleaves eIF-4G, but the fact that coxsackievirus and rhinovirus 2A\textsuperscript{pro} directly cleave this initiation factor (Lamphear et al., 1993; Haghighat et al., 1996) makes it doubtful that a cellular protease activated by 2A\textsuperscript{pro} is responsible for eIF-4G cleavage (Wyckoff et al., 1990, 1992). Poliovirus 2A\textsuperscript{pro} is a multifunctional enzyme; in addition to proteolysis of viral precursors and eIF-4G, 2A\textsuperscript{pro} stimulates poliovirus RNA translation (Hambidge & Sarnow, 1992) and RNA replication (Yu et al., 1995; Lu et al., 1995) by mechanisms that are poorly understood. An interaction between 2A\textsuperscript{pro} and the internal ribosome entry site (IRES), together with an active role for the C-terminal cleavage product of eIF-4G, may be involved in the mechanism of transactivation of viral mRNA translation by 2A\textsuperscript{pro} (Hambidge & Sarnow, 1992; Macadam et al., 1994; Ziegler et al., 1995; Ventoso & Carrasco, 1995).

Poliovirus 2A\textsuperscript{pro} is very toxic to yeast cells (Barco & Carrasco, 1995a). This toxicity has been exploited to develop a genetic system to obtain 2A\textsuperscript{pro} mutants (Barco et al., 1997). The molecular target of poliovirus 2A\textsuperscript{pro} in yeast cells remains to be determined, although translation seems not to be affected by the protease (Barco & Carrasco, 1995 a). It would be of interest to reproduce in yeast cells the different 2A\textsuperscript{pro} activities, such as cleavage of eIF-4G or the transactivation of translation of mRNAs containing the poliovirus IRES. Attempts to express poliovirus proteins from full-length poliovirus cDNA in yeast have so far been unsuccessful, despite the fact that poliovirus cDNA can be transcribed to form an apparently genuine viral RNA (Coward & Dasgupta, 1992). The defect in the expression of poliovirus RNA in yeast cells is located at the level of initiation of translation, which is blocked by the presence of a small RNA inhibitor (Coward & Dasgupta, 1992; Das et al., 1994). These authors suggest that this small RNA interacts directly with the cellular translational machinery that binds to the poliovirus IRES (Coward & Dasgupta, 1992; Das et al., 1994). However, several groups have shown that cap-independent translation can be performed by the yeast translation apparatus, at least in cell-free systems (Allmann et al., 1990; lizuka et al., 1995; lizuka & Sarnow, 1997).

We have used the yeast \textit{Saccharomyces cerevisiae} to analyse the interaction between human eIF-4G and poliovirus 2A\textsuperscript{pro}, and to test the effect of co-expression of these proteins on two different reporter constructs: the luciferase gene with or without a poliovirus IRES at the 5′ terminus.

### Methods

**Microbial strains.** \textit{E. coli} DH5\textsuperscript{+} (Sambrook et al., 1989) was used for the construction of all expression plasmids. The \textit{S. cerevisiae} strains used were W303-1B (MAT\textalpha\, ade2\, his3\, leu2\, trp1\, ura3), YCG165, YCG209, YCG212 and CBY12 (Goyer et al., 1993), and were generously provided by M. Allmann (Universität Bern, Bern, Switzerland).

**Yeast media, growth, transformation and induction.** Yeast cells were selected and grown in minimal YNB medium containing 0.67% yeast nitrogen base without amino acids, and 2% of either glucose (YNB.Glu), galactose (YNB.Gal) or lactic acid (YNB.Lac, pH 6.5), plus 20 mg/l of the required amino acid or bases dictated by the auxotrophic markers. For induction, 2% galactose was added directly to YNB.Lac. After the addition of galactose this medium was referred to as YNB.Gal (Barco & Carrasco, 1995b). Transformation of yeast by the lithium acetate procedure was performed as previously described (Rose et al., 1990).

**Plasmid constructions.** Construction of vectors encoding luciferase or the different poliovirus proteins to be expressed in yeast was carried out by standard procedures (Sambrook et al., 1989). The plasmids used were based on the yeast–\textit{E. coli} shuttle vector pEMBLxyx4 (Cesareni & Murray, 1987). The sequences L1.luc and luc were obtained as previously described (Ventoso & Carrasco, 1995). Plasmid pEMBL.luc encodes luciferase, whereas pEMBL1.luc contains the complete poliovirus 5′ leader region upstream of the luciferase ORF. Plasmids pEMBL2A, pEMBL2B, pEMBL2C, pEMBL2BC, pEMBL2BC128 and pEMBL3C encode the poliovirus proteins indicated, with an additional methionine at the N terminus as described previously (Barco & Carrasco, 1995c). Constructs were confirmed by DNA sequencing using the dideoxy method (Sambrook et al., 1989). Plasmids KS.luc and KS5′ ‘Leader containing the luciferase ORF or the poliovirus 5′ leader region were used to obtain radiolabelled RNA probes by \textit{in vitro} transcription with T3 RNA polymerase. To co-express human eIF-4G and poliovirus proteins, the constitutive plasmid pYcDE8 was used (Klein & Roof, 1988), and was generously provided by R. Klein (Upjohn, Kalamazoo, MI, USA). Plasmid pSK-HFC1 (Joshi et al., 1994), generously supplied by R. E. Rhodes (Louisiana State University Medical Center, Baton Rouge, LA, USA), was digested with EcoRI, and the fragment bearing the complete ORF of eIF-4G was cloned in the EcoRI site of pYcDE8, giving rise to plasmid pYc.p220.

**Luciferase assay and protein determination.** Extracts were prepared from harvested cells resuspended in luciferase reaction buffer minus ATP (25 mM glycyglycine, pH 7.8, 15 mM MgSO\textsubscript{4}, 1 mM DTT) and ground three times for 1 min each using glass beads. The lysate was centrifuged for 10 min at 14000 r.p.m. and the pellet discarded. The supernatant used in the luciferase assay was obtained and tested as described elsewhere (Ventoso & Carrasco, 1995). The Bio-Rad protein assay was used to determine protein concentration. Yeast extracts for Western blot analysis were prepared as previously indicated (Yaffe & Schatz, 1984). Western blotting was carried out as previously described (Barco & Carrasco, 1995b).

**Northern blot analysis.** Total yeast RNA was prepared using the phenol–LiCl method (Rose et al., 1990). We have used both radiolabelled and biotinylated riboprobes. Synthesis of both kinds of riboprobe was carried out as recommended by the manufacturer (Amersham and Clontech, respectively). Northern blot analysis and hybridization assays were performed as described previously (Sambrook et al., 1989). When biotinylated riboprobes were used we adapted the dot blot protocol described by Ventoso & Carrasco (1995).

### Results

**Inducible expression of luciferase in yeast cells: influence of the poliovirus 5′ leader region**

Poliovirus mRNA possesses a long 5′ untranslated leader region (5′ UTR) of 740 nt that allows mRNAs to be translated by internal initiation (Sonenberg, 1990; Ehrenfeld, 1996). A portion of the 5′ UTR, encompassing approximately nucleo-
tides 130–600, is sufficient to direct initiation complexes to bind internally to the IRES (Ehrenfeld, 1996). The functionality of the poliovirus IRES in yeast cells has been controversial. In vitro translation of mRNAs bearing the picornavirus IRES has been achieved by some groups (Altmann et al., 1990; Iizuka et al., 1995; Iizuka & Sarnow, 1997), while no translatability either in yeast cells or in cell-free systems has been reported by others (Coward & Dasgupta, 1992; Das et al., 1994; Russell et al., 1991; Evstafieva et al., 1993). To analyse the functionality of the poliovirus IRES in S. cerevisiae we constructed two plasmids, pEMBL.luc and pEMBL.L1.luc, bearing the luciferase gene under a galactose-inducible promoter. One of these constructs contained the poliovirus 5′ UTR linked to the coding sequence of luciferase (pEMBL.L1.luc). Expression of luciferase was repressed in glucose-containing medium, while there was a 50- to 100-fold stimulation of luciferase activity when yeast containing pEMBL.luc was induced in galactose medium (Fig. 1a). The presence of the poliovirus 5′ UTR (pEMBL.L1.luc) was clearly detrimental for luciferase expression, although some luciferase was synthesized in galactose medium (about 10-fold stimulation). Therefore, the synthesis of luciferase from pEMBL.L1.luc was clearly lower as compared to expression from the luciferase gene without the poliovirus 5′ UTR.

Although luciferase activity was lower in yeast cells bearing the poliovirus 5′ UTR, the protein might have been synthesized as an inactive or truncated form of the enzyme. To address this possibility immunoblot analysis of the proteins present in cells was carried out using a luciferase-specific antibody. Our results reveal that after galactose induction luciferase protein was not apparent in yeast cells bearing the poliovirus 5′ UTR (Fig. 1b).

Another possibility to account for the low level of luciferase activity in yeast cells containing pEMBL.L1.luc was that transcription of the luciferase gene was poor as compared to cells bearing pEMBL.luc. To address this issue, mRNAs from both cell types grown in glucose or galactose medium were isolated and hybridized with specific probes directed against the luciferase coding region or the poliovirus 5′ UTR. Luciferase mRNA bearing the poliovirus 5′ UTR (Leader-luc mRNA) was detectable at levels comparable to luciferase mRNA (Fig. 2a). As a control, the luciferase activity present in these cells was measured before mRNA extraction (Fig. 2b). In conclusion, our results indicate that the luciferase gene containing the poliovirus 5′ UTR was transcribed in yeast cells upon galactose induction, but the Leader-luc mRNAs made were very poorly translated.

**Co-expression of human eIF-4G and poliovirus 2Apro in yeast**

Poliovirus 2Apro enhances translation of mRNAs containing the poliovirus 5′ UTR both in human cells and in cell-free systems (Hambidge & Sarnow, 1992; Novoa et al., 1997). This transactivation effect is thought to be mediated by the selective cleavage of initiation factor eIF-4G (Lamphear et al., 1995). The participation of intact eIF-4F complex, or even the C terminus of eIF-4G bound to eIF-4A, in the internal initiation of mRNAs bearing the picornavirus IRES is now well-established (Ohlmann et al., 1996, 1997; Lamphear et al., 1995; Pestova et al., 1996). Accordingly, we thought it of interest to analyse the functionality of Leader-luc mRNA in yeast cells expressing the human eIF-4G or in cells that co-expressed eIF-4G together with poliovirus 2Apro. The isolation of yeast cells expressing the three heterologous proteins luciferase, human eIF-4G and poliovirus 2Apro poses several technical problems. As an initial step toward this goal, the gene encoding human eIF-4G was subcloned under the control of a constitutive promoter present in the plasmid pYcDE8, which complements tryptophan auxotrophy (TRP1), while poliovirus 2Apro was subcloned in pEMBLyex4 (the same plasmid used to subclone the luciferase gene) containing the genes URA3 and LEU2. Yeast cells were co-transfected with plasmids pYc.p220 and pYc.p220.
Fig. 2. Luciferase transcripts present in yeast cells. (a) Northern blot analysis of RNA obtained from yeast transformed with pEMBL.yex4 (V), pEMBL.luc (luc) or pEMBL.L1.luc (L1), after 16 h growth in YNB.Glu (Glu) or YNB.Gal (Gal) medium. Two radiolabelled RNA probes were used: luc Probe RNA is complementary to luciferase positive mRNA and 5’ Polio Probe is complementary to the 5’ UTR of the poliovirus positive sequence. The probes and the assay were performed as indicated in Methods. (b) Luciferase activity after 16 h growth in YNB.Glu or YNB.Gal in yeast cells transformed with pEMBL.luc (hatched bars) or pEMBL.L1.luc (filled bars).

pEMBL.2A, and cells that expressed both eIF-4G and poliovirus 2A\(^{pro}\) were isolated. The constitutive expression of eIF-4G had no apparent effect on cell growth. These yeast cells inducibly synthesized poliovirus 2A\(^{pro}\) when placed in galactose medium. Expression of eIF-4G was easily detected by Western blotting using rabbit polyclonal antibodies (Fig. 3a). Cleavage of eIF-4G readily occurred in yeast cells induced to synthesize poliovirus 2A\(^{pro}\) (Fig. 3a). Only the C-terminal moiety of eIF-4G was detected, while the N-terminal fragments were not apparent. Although we have no explanation for the lack of N-terminal fragments after eIF-4G cleavage, it might be that these polypeptides are unstable in yeast and they were further degraded. Nevertheless, our findings clearly illustrate that poliovirus 2A\(^{pro}\) is able to recognize and cleave eIF-4G in yeast cells. Moreover, if eIF-4G is indeed cleaved in human cells by a cascade mechanism as suggested (Wyckoff \textit{et al}., 1992), our findings indicate that the putative enzymes involved in this cascade are also present in yeast. Another possibility that has not yet been proven is that poliovirus 2A\(^{pro}\) directly proteolysed eIF-4G both in yeast and human cells, as occurs with other picornavirus proteases (Lamphear \textit{et al}., 1993; Haghighat \textit{et al}., 1996).

Our antibodies against human eIF-4G do not recognize the yeast counterpart of eIF-4G, also known as p150. To test if yeast p150 was also recognized and cleaved by poliovirus 2A\(^{pro}\), immunoblot analysis using a p150-specific antibody was carried out both with control cells and with cells that synthesized 2A\(^{pro}\). Our findings indicate that yeast eIF-4G was not degraded by poliovirus 2A\(^{pro}\) (Fig. 3b). These results are in good agreement with the observation that rhinovirus 2A\(^{pro}\) does not cleave yeast p150 (Klump \textit{et al}., 1996).

Since some initiation factors are interchangeable between
Poliovirus 2A cleaves human eIF-4G in yeast

The transactivation of the poliovirus IRES by 2A<sup>pro</sup> requires cleavage of eIF-4G (Hambidge & Sarnow, 1992; Ventoso & Carrasco, 1995). The availability of yeast cells expressing luciferase linked to an IRES and containing poliovirus 2A<sup>pro</sup> and human eIF-4G would provide a system to assay transactivation in this micro-organism. Such a system would be more amenable to genetic studies of the molecular mechanisms of transactivation. The simultaneous expression of the three genes encoding luciferase, poliovirus 2A<sup>pro</sup> and eIF-4G was achieved by using diploid yeast cells. Thus, the yeast strain FYBL1 (MATα) was transformed with plasmids pEMBLyex4 (control plasmid) or pEMBL.2A, while yeast strain W303–1B (MATα) was co-transformed with plasmid pYCDE8 (control plasmid) or pYc.p220 in combination with each one of the following plasmids: pEMBLyex4 (control plasmid), pEMBL.luc and pEMBL.L1.Luc. All the diploids obtained by mating the corresponding haploid strains were assayed for luciferase activity at different times after galactose induction (Fig. 5).

The levels of luciferase activity measured in diploid yeast cells bearing pEMBL.luc or pEMBL.L1.luc were comparable to those found previously for cells containing only these plasmids (compare Fig. 1 and Fig. 5). On the other hand, yeast containing pEMBL.2A showed growth inhibition when the 2A<sup>pro</sup> gene was induced with galactose, in agreement with the finding that 2A<sup>pro</sup> is cytotoxic to yeast. Finally, the presence of human eIF-4G in diploid cells containing pYc.p220 was detected by Western blotting (Fig. 5b). As previously observed with the haploid yeast strain (Fig. 3a), eIF-4G was cleaved in cells containing pEMBL.2A and grown in galactose medium (Fig. 5b). These results indicated the feasibility of obtaining diploid yeast strains that expressed the three heterologous genes.

As regards the action of poliovirus 2A<sup>pro</sup> or human eIF-4G, either alone or in combination, on luciferase expression from pEMBL.luc or pEMBL.L1.luc (Fig. 5a), a number of conclusions

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Fig. 4. Human eIF-4G does not complement yeast eIF-4G deficiency. Immunoblot analysis of yeast extracts from the strains indicated transformed with pYcDE8 (Yc) or pYc.p220 (p220) with antiserum against yeast (a) or human (b) eIF-4G. YCG165 cells were grown in YNB.Glu medium and CBY12 cells in YNB.Gal medium (Gal). In order to test for inhibition of growth resulting from depletion of eIF-4G in CBY12 cells, an aliquot of these cells was grown in YNB.Glu medium; note the reduced level of yeast eIF-4G after 5 h in this medium (Glu (5 h)). The positions of yeast eIF-4G (p150), of human eIF-4G (p220) and its cleavage products (CP) and of molecular mass markers are indicated. (c) Growth of CBY12 cells bearing pYcDE8 or pYc.p220. Cells were streaked onto plates containing YNB.Gal medium (YNB-GAL), which induced yeast eIF-4G synthesis, or YNB.Glu medium, which did not. Cells were streaked onto YNB.Glu plates from a YNB.Glu plate (YNB-GLU 1) or from a YNB.Glu plate (YNB-GLU 2).

yeast and mammalian cells (Altmann et al., 1989; Naranda et al., 1994; Merrick & Hershey, 1996), it was of interest to analyse whether this also applied to eIF-4G. Such complementarity would permit genetic manipulation of human eIF-4G in S. cerevisiae. For this experiment yeast cells (YCG165) defective in the gene encoding the principal p150 isoform were transformed with pYc.p220 encoding the human eIF-4G. Cells isolated that constitutively expressed human eIF-4G showed the same growth defect as the YCG165 strain (results not shown). In addition, yeast cells doubly disrupted in the two p150 genes, and containing the principal p150 gene isofrom under a galactose-inducible promoter (strain CBY12), were also transformed with pYc.p220. Fig. 4 shows that YCG165 cells are indeed deficient in p150 (Fig. 4a) and that both YCG165 and CBY12 contain human eIF-4G (Fig. 4b). Once again no alleviation of the growth defect by human eIF-4G was observed (Fig. 4c). Therefore, human eIF-4G failed to complement yeast initiation factor p150. eIF-4G interacts with eIF-4E, eIF-3 and eIF-4A. The first two factors can be interchanged between mammalian and yeast cells (Altmann et al., 1989; Naranda et al., 1994), but the last one cannot (Prat et al., 1990). The lack of eIF-4G/eIF-4A binding could explain this negative result.
can be drawn. Some stimulation of luciferase activity was found when intact human elf-4G was present, particularly when luciferase was synthesized from mRNAs bearing the poliovirus IRES. Moreover, poliovirus 2Apro strongly inhibited luciferase synthesis (10-fold inhibition) from either pEMBL.luc or pEMBL.L1.luc, irrespective of the presence or absence of elf-4G (Fig. 4a, b).

To clarify whether the block in luciferase expression was located at the transcriptional level, RNA was extracted from the diploid yeast strains after 5.5 h galactose induction. Fig. 6 shows that poliovirus 2Apro strongly reduced the level of luciferase transcripts from cells containing either pYcDE8 or pYc.220 and pEMBL.luc or pEMBL.L1.luc. Therefore, 2Apro is a potent inhibitor of transcription in yeast cells. These findings support our previous results on the inhibition of uridine incorporation in yeast cells that express poliovirus 2Apro (Barco & Carrasco, 1995a). In addition, these results agree with previous experiments indicating that transcription of a reporter gene was depressed by poliovirus 2Apro in mammalian cells (Davies et al., 1991).

To test the specificity of the inhibition of luciferase expression by 2Apro the action of other poliovirus proteins was analysed. To this end, the genes encoding nonstructural proteins 2B, 2C, 2BC, 3C, and a 2BC deletion mutation (2BC128) that is highly cytotoxic to yeast, were co-expressed with the luciferase gene with or without the poliovirus 5′ UTR. The synthesis of both luciferase and each individual poliovirus protein was triggered by incubation in galactose medium. Apart from 2Apro, only 2BC and its deletion mutant 2BC128 showed any inhibition of luciferase activity (results not shown). These findings indicate that the two poliovirus nonstructural proteins that interfere with yeast growth, 2Apro and 2BC, also block luciferase expression, although 2BC is less inhibitory than 2Apro. Nevertheless, our results indicate that expression of any poliovirus protein, including the protease 3Cpro, is not necessarily detrimental to luciferase expression in yeast.

**Regions of poliovirus 2Apro involved in elf-4G cleavage and luciferase inhibition in *S. cerevisiae***

The fact that poliovirus 2Apro is cytotoxic to yeast raised the possibility of selecting 2Apro variants, generated by random mutagenesis, which are devoid of inhibitory activity
Poliovirus 2A cleaves human eIF-4G in yeast

Fig. 7. Luciferase activity in yeast cells expressing 2Apro variants. (a) Primary sequence of poliovirus 2Apro. The putative catalytic triad is shown in outline type (residues 20, 38 and 109), and the residues mutated (Barco et al., 1997) are boxed and in bold type. (b) Luciferase activity in diploid yeast cells transformed with pYc.p220, pEMBL.luc, and one of pEMBL.2A (2A) or the different pEMBL.2A* plasmids shown. The amino acid changes are indicated. Cells were grown in YNB.LGal medium and extracts were prepared 6 h after induction and used in the luciferase assay as indicated in Methods. (c) Cleavage of human eIF-4G in the yeast cells described in (b). Crude extracts were prepared 6 h after induction, separated by SDS–PAGE, transferred to nitrocellulose membranes and immunoreacted with antiserum against human eIF-4G. Extracts from uninfected HeLa cells (-) or poliovirus-infected HeLa cells (+) were used as controls. The positions of human eIF-4G (p220) and its cleavage products (CP) are indicated.

Luciferase activity was monitored 6 h after induction of luciferase and 2A* genes. Some of the 2Apro variants, such as 2, 5 and 14, did not affect luciferase expression, while variants 7, 8, 12, 17 and 19 were as inhibitory as wt 2Apro (Fig. 7b). The other variants showed intermediate phenotypes. These findings indicate that although all the 2Apro mutants were selected by their failure to block yeast growth, they led to a variety of phenotypes when inhibition of the expression of a particular gene was assayed.

The co-expression of the 2Apro variants with eIF-4G provided an interesting result: only the mutants that generated viable polioviruses in our previous work (Barco et al., 1997) cleaved eIF-4G in yeast (Fig. 7c). It should be remembered that most of the 2Apro variants cleaved eIF-4G in human cells (Barco et al., 1997). These differences do not rely on the different constructs used in the case of yeast and human cells, because this was observed even when pEMBL.2A* was transfected in COS cells and expressed with vaccinia virus (Barco et al., 1997). A number of explanations can be put forward to account for these findings. One possibility is that eIF-4G is cleaved more efficiently by 2Apro when bound to eIF-4E, as has been reported for rhinovirus 2Apro. Thus, eIF-4G may be more susceptible to cleavage by the different 2Apro variants when present as part of the eIF-4F complex, a situation that occurs in mammalian cells. Since human eIF-4G does not complement p150 (see above results) the human factor may
remain uncomplexed in yeast cells, without forming part of yeast eIF-4F, and could be cleaved only by the most active 2A<sup>pro</sup> variants, those that generate viable polioviruses. Another explanation is that the structural conformation of free unbound eIF-4G in yeast and mammalian cells differs, being susceptible to cleavage in yeast cells only by some 2A<sup>pro</sup> variants with higher proteolytic activity.

**Discussion**

The usefulness of *S. cerevisiae* for determining the exact mode of action of animal virus proteins has recently been documented for several poliovirus proteins. Thus, protein 2BC promotes the formation of intracellular vacuoles and permeabilizes yeast cells in a manner akin to that found in mammalian cells (Barco & Carrasco, 1995b, 1998). In addition, the high toxicity of poliovirus 2A<sup>pro</sup> to yeast has been exploited to identify genetic variants of 2A<sup>pro</sup> devoid of growth inhibitory properties (Barco et al., 1997). Some of these variants show defects in substrate cleavage activities and/or in their capacity to enhance translation of poliovirus RNA (Barco et al., 1997). In this work we focussed on the cleavage activity of poliovirus 2A<sup>pro</sup> towards human eIF-4G and asked the question 'does 2A<sup>pro</sup> stimulate translation in yeast cells of mRNAs bearing the poliovirus 5′ UTR?'. As an initial step in this direction the translatability of an mRNA bearing the poliovirus IRES in yeast was tested. The pioneering work of Altmann et al. (1990) indicated that the poliovirus IRES was functional in cell-free systems derived from yeast. These findings have been verified by P. Sarnow's group (Iizuka et al., 1995; Iizuka & Sarnow, 1997). However, these results contrast with the low translatability of poliovirus RNA electroporated into yeast spheroplasts (Russell et al., 1991). In agreement with this last result Coward & Dasgupta (1992) have reported that poliovirus RNA is not functional either in cell-free systems or in yeast cells. The lack of functionality of the poliovirus IRES stems from the presence of a small RNA in yeast cells that may selectively block translation of mRNAs bearing the poliovirus 5′ UTR (Das et al., 1994). This small RNA is inhibitory even for poliovirus mRNA translation in HeLa cells (Das et al., 1994). Our present findings agree with the idea that mRNAs containing the poliovirus 5′ UTR show low translatability in *S. cerevisiae*, despite the presence of significant amounts of the Leader-luc mRNA.

Since poliovirus mRNA is translated much better (about 10-fold stimulation) in the presence of poliovirus 2A<sup>pro</sup> when eIF-4G has been degraded in human cells (Hambidge & Sarnow, 1992; Ventoso & Carrasco, 1995), it was of interest to assay the action of 2A<sup>pro</sup> on the translation of Leader-luc mRNA in yeast. Moreover, several laboratories have established that the C-terminal moiety of eIF-4G participates in picornavirus RNA translation (Hambidge & Sarnow, 1992; Ziegler et al., 1995). Therefore, we examined the transactivation of Leader-luc mRNA in cells co-expressing poliovirus 2A<sup>pro</sup> and human eIF-4G. Our results indicate that the presence of poliovirus 2A<sup>pro</sup> and cleaved eIF-4G does not transactivate the translation of luciferase mRNA bearing the poliovirus 5′ UTR in yeast cells. Since transactivation in mammalian cells involves eIF-4A bound to the C-terminal fragment of eIF-4G, the lack of transactivation in yeast may be due to the failure of human eIF-4G to interact with yeast eIF-4A. Although it now appears that the eIF-4G clone provided by R. E. Rhoad's group and used in this work lacks the N terminus (Gradi et al., 1998), this clone was active in a number of *in vitro* assays (Joshi et al., 1994). Also, only the C-terminal fragment has been reported to be involved in transactivation. Moreover, recent results indicate that an additional form of eIF-4G exists in human cells (Gradi et al., 1998). Thus, the possibility that the untruncated form of eIF-4G or even the second isoform described is able to complement p150 deficiency and/or to transactivate an IRES in yeast cells remains open.

As a preliminary step in this study, yeast cells that synthesize both poliovirus 2A<sup>pro</sup> and human eIF-4G were obtained. Notably, 2A<sup>pro</sup> efficiently cleaved human eIF-4G in yeast, and the C terminus of this factor, thought to participate in transactivation (Lamphear et al., 1995; Ohlmann et al., 1995; Pestova et al., 1996), was stable upon cleavage. This finding suggests that poliovirus 2A<sup>pro</sup> may interact with and cleave human eIF-4G directly, as occurs with other picornavirus proteases (Lamphear et al., 1993; Haghighat et al., 1996). Alternatively, if poliovirus 2A<sup>pro</sup> degrades human eIF-4G through the activation of other proteases (Wyckoff et al., 1990, 1992) it should be inferred that these putative proteases are also present in yeast and display the same specificity for cleavage of eIF-4G as their human counterparts. A third possibility is that poliovirus 2A<sup>pro</sup> is able to proteolyse eIF-4G directly and other proteases may act subsequently on the eIF-4G fragments generated. In the case of yeast cells the N-terminal moiety would be further degraded to fragments not recognized by our antibodies, while a heterogeneous collection of fragments would appear in human cells. In fact, it remains unknown whether the different N-terminal fragments of eIF-4G that appear as a result of 2A<sup>pro</sup> activity in human cells are due to factor heterogeneity or to further protease activity. At any rate, the fact that human eIF-4G is cleaved by poliovirus 2A<sup>pro</sup> provides a means to assay variants of 2A<sup>pro</sup> devoid of this activity, as shown here, or to test a number of eIF-4G variants potentially resistant to cleavage.

As regards the mode of action of 2A<sup>pro</sup> in yeast cells, our present findings indicate that transcription is certainly a target of this protease, thus accounting for the cytotoxicity observed initially with poliovirus 2A<sup>pro</sup> in yeast (Barco & Carrasco, 1995a). Previous work showed that the yeast translation initiation factor p150 was not degraded by rhinovirus 2A<sup>pro</sup> (Klump et al., 1996). This finding is corroborated by our work using poliovirus 2A<sup>pro</sup>. It will be of interest now to identify the component of yeast transcription that is the target of 2A<sup>pro</sup>. In...
this respect, poliovirus 2A<sup>pro</sup> may be a useful tool to inactivate selectively that component in order to analyse in detail its function in yeast transcription.

In summary, several conclusions can be drawn from our findings. One is that the poliovirus IRES is not functional in yeast cells. This lack of function cannot be rescued by the presence of poliovirus 2A<sup>pro</sup>, of intact human eIF-4G or of 2A<sup>pro</sup> plus the C terminus of eIF-4G. Second, poliovirus 2A<sup>pro</sup> actively cleaves eIF-4G in yeast, representing a useful system to analyse this proteolytic activity in a micro-organism such as <i>S. cerevisiae</i>. Finally, poliovirus 2A<sup>pro</sup> blocks transcription in yeast cells; the transcription factor inactivated by this protease remains to be identified.

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