The GCD10 subunit of yeast eIF-3 binds the methyltransferase-like domain of the 126 and 183 kDa replicase proteins of tobacco mosaic virus in the yeast two-hybrid system

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The tobacco mosaic virus (TMV) replicase complex contains virus- and host-encoded proteins. In tomato, one of these host proteins was reported previously to be related serologically to the GCD10 subunit of yeast eIF-3. The yeast two-hybrid system has now been used to show that yeast GCD10 interacts selectively with the methyltransferase domain shared by the 126 and 183 kDa TMV replicase proteins. These findings are consistent with a role for a GCD10-like protein in the TMV replicase complex and suggest that, in TMV-infected cells, the machinery of virus replication and protein synthesis may be closely connected.

The replication of positive-stranded RNA viruses is carried out by an RNA-dependent RNA polymerase that comprises part of a replicase complex containing virus- and host-encoded proteins (Buck, 1996). The tobacco mosaic virus (TMV) replicase contains two virus-encoded subunits of 126 and 183 kDa that are required for efficient RNA replication (Ishikawa et al., 1986, 1991). Both of these proteins are translated from the 5′-proximal open reading frame of the TMV genomic RNA, the 183 kDa protein being synthesized by occasional readthrough of the leaky termination codon of the 126 kDa coding sequence (Pelham, 1978). Within these proteins, three functional domains have been identified (Haseloff et al., 1984; Hodgman, 1988; Koonin & Dolja, 1993). The two proteins share an N-terminal methyltransferase-like domain that has been shown to have guanylyltransferase activity (Dunigan & Zaitlin, 1990) and a second domain that has sequence similarity to many viral and cellular helicases (Gorbalenya et al., 1988). The readthrough region of the 183 kDa protein contains amino acid sequence motifs common to many polymerases, including the Mg²⁺-binding GDD motif (Argos, 1988). This polymerase-like domain is also present in the putative 54 kDa protein (Golemboski et al., 1990). However, despite the presence of a sub-genomic RNA encoding this protein in TMV-infected tobacco leaves (Sulzinski et al., 1985), the 54 kDa protein has never been detected in virus-infected tissue (Carr et al., 1992).

Host proteins were first implicated in the replication of positive-stranded RNA viruses by the discovery that the replicase of the bacteriophage Qβ contains four host-encoded proteins: the S1 30S ribosomal protein, a ribosome-associated protein termed HF-1 and two protein synthesis elongation factors, EF-Tu and ET-Ts (Blumenthal & Carmichael, 1979). Host proteins have also been found that co-purify with the replicase of brome mosaic virus (BMV) (Quadt & Jaspars, 1990), cowpea mosaic virus (Dorssers et al., 1984), cucumber mosaic virus (Hayes & Buck, 1990), red clover necrotic mosaic virus (Bates et al., 1995), Sindbis and Semliki Forest viruses (Barton et al., 1991) and turnip yellow mosaic virus (Mouches et al., 1984). So far, the identities and role(s) in virus replication of most of these host proteins remain elusive. However, there is evidence to suggest that subunits of the eukaryotic translation initiation factor eIF-3 play a role in the replication of BMV (Quadt et al., 1993) and TMV (Osman & Buck, 1997).

In the case of TMV, several host proteins have been found to be associated with replicase preparations purified from infected tissue (Osman & Buck, 1997; Watanabe et al., 1999). Osman & Buck (1997) identified a 56 kDa protein, immunologically related to the GCD10 subunit of yeast eIF-3, in the TMV replicase isolated from infected tomato tissue. The viral 126 and 183 kDa proteins, plus the host-encoded 56 kDa protein, could be purified by using either antibodies against the TMV-L 126 kDa protein or antibodies against yeast GCD10 (Osman & Buck, 1997). The 56 kDa host protein could not be observed by SDS–PAGE analysis of an immuno-purified replicase preparation from tissue infected with TMV-OM because of the large amounts of IgG present (Watanabe et al., 1999). However, a role for the GCD10-like protein in virus replication is very likely, since the synthesis of TMV genomic
ssRNA and dsRNA by the TMV-L replicase was inhibited by antibodies against the GCD10 protein, and this inhibition could be prevented by competition with purified yeast GCD10 protein (Osman & Buck, 1997).

It is not known whether the plant GCD10-like protein binds the virus-encoded components of the TMV-L replicase complex directly or whether the interaction is via a bridging factor, perhaps another component of the translation initiation apparatus. Since the plant GCD10-like protein has not yet been cloned, we decided to use the yeast two-hybrid system to determine whether the yeast GCD10 protein interacts directly with the TMV-encoded replicase proteins and, if so, which domain(s) of the 126/183 kDa proteins is involved in the interaction.

By using a full-length TMV-L cDNA clone (Ohno et al., 1984) as a template, PCR was used to generate cDNA fragments corresponding to the three putative functional domains of the TMV 126/183 kDa protein sequence (Fig. 1). The methyltransferase-like (designated Dom 1 in Fig. 1) and helicase-like (Dom 2) domains in this study span amino acids 1–484 and 647–1116, respectively (Fig. 1), and correspond to the boundaries determined by primary amino acid comparisons between plant-infecting members of the alphavirus-like superfamily and Sindbis virus (Ahlquist et al., 1985). The polymerase-like domain (Dom 3) contained the amino acids 1141–1616 of the 183 kDa readthrough region, which is equivalent to the putative 54 kDa protein open reading frame (Sulzinski et al., 1985). The DNA sequences were cloned into the two-hybrid vectors pAS2 and pACTII to generate protein fusions with the GAL4 DNA-binding domain and transcription-activation domain, respectively (Harper et al., 1993; Li et al., 1994). All amplified DNA fragments were gel-purified, digested with appropriate restriction enzymes, ligated into pAS2 and pACTII and checked by automated DNA sequencing before use in the yeast two-hybrid system. A DNA clone pMG107 (Garcia-Barrio et al., 1995) encoding the yeast GCD10 protein sequence was excited with BamHI and Xhol and sub-cloned into pACTII cut with the same restriction enzymes to yield pACTIIpGCD10. To sub-clone into pAS2, the GCD10 coding sequence in pMG107 was digested with BamHI and PstI and the fragment was ligated to produce pAS2GCD10. Cells of Saccharomyces cerevisiae strain Y190 (Harper et al., 1993) were transformed by the method of Gietz & Schiestl (1995) and colonies were selected on medium lacking tryptophan and leucine. An interaction between two fusion proteins was identified by induction of β-galactosidase activity. β-Galactosidase activity was identified in the first instance by using a filter colony lift assay with X-Gal as a substrate (Breeden & Nasmyth, 1985) and was subsequently confirmed by a spectrophotometric liquid assay with yeast cell extracts with o-nitrophenyl β-D-galactopyranoside as a substrate (Faure et al., 1998).

Our results indicate that the GCD10 protein interacts with the methyltransferase-like domain of the TMV-L 126/183 kDa proteins (Table 1). The interaction was found to be orientation-specific. That is to say, the interaction between GCD10 and the TMV 126/183 kDa methyltransferase domain was only observed with the two-hybrid vector combination pAS2GCD10–pACTIIDom1 (Table 1; Fig. 2). This result was confirmed spectrophotometrically by using extracts of transformed yeast. In three separate experiments, we found that the level of β-galactosidase activity in cells transformed with the vector combination pAS2GCD10–pACTIIDom1 was a minimum of fivefold greater in all cases than that in cells transformed with the vector combination pAS2Dom1–pACTIIGCD10 or in untransformed yeast (data not shown). It was also found that Y190 cells containing the vector combination pAS2GCD10–pACTIIDom1 grew well on medium lacking histidine, tryptophan and leucine but containing 25 mM 3-aminotriazole (Yocum et al., 1984) (data not shown).

### Table 1. Two-hybrid analysis of the interactions of the GCD10 protein with virus domains

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<tr>
<th>Plasmids in strain Y190*</th>
<th>Colony appearance†</th>
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<td>pAS2</td>
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* Domains are defined in the legend to Fig. 1. —, Plasmids without inserts.
† Colony appearance in the filter assay is given as: w, white (no detectable β-galactosidase activity); b, blue (detectable β-galactosidase activity). Filter assays were performed at least three times, each time with separately transformed yeast lines.
This indicated that the second reporter gene his3 was induced in these cells and further confirmed the interaction.

Similar 'directionality' is not unusual with the yeast two-hybrid system (Estojak et al., 1995). We suspect that the methyltransferase-like domain fails to fold correctly when fused to the DNA-binding domain of the GAL4 protein. In support of this, we have observed that the methyltransferase-like domain interacts with another putative host factor in the yeast two-hybrid system when fused to the DNA-activation domain of GAL4 (pACTIIDom1), but not when fused to the DNA-binding domain of GAL4 (pAS2Dom1) (Taylor, 1999). Yeast two-hybrid analysis showed that the interaction of the GCD10 protein with the 126/183 kDa protein of TMV is specific for the methyltransferase-like domain, since the GCD10 protein did not interact with the virus helicase- or polymerase-like domains (Table 1). There was no evidence that the pACTIIDom1 construct could induce transcription by itself. No β-galactosidase activity was seen in singly transformed yeast cells or in cells transformed with pACTIIDom1 plus empty pAS2, or together with pAS2 containing Dom1 itself or an unrelated sequence (Taylor, 1999).

We also carried out experiments to determine whether the cloned domains (Fig. 1) of the 126 and 183 kDa proteins were able to interact with each other to form homodimers or heterodimers. However, we found no evidence for dimerization (data not shown). This is consistent with work with BMV, in which the 1a helicase-like domain interacted with the 2a polymerase-like protein (Smirnyagina et al., 1996) but did so via an interaction with a region of 115 amino acids that lies upstream of the conserved polymerase-like domain (O'Reilly et al., 1998) and not with the polymerase-like domain itself (Kao & Ahlquist, 1992; O'Reilly et al., 1995). The 115 amino acid region, necessary and sufficient for the 1a–2a interaction in BMV, is absent from the TMV 126/183 kDa protein sequence. This is not surprising, since the BMV 1a–2a complex is the functional equivalent of the TMV 183 kDa protein, where the methyltransferase- helicase- and polymerase-like domains are found within a single polypeptide chain. However, we cannot exclude the possibility that our results are due to the misfolding of the domains when fused with the GAL4 activation or DNA-binding moieties.

Recently, Watanabe et al. (1999) used antibodies specific for either the RNA polymerase (anti-P) or the methyltransferase (anti-M) domains of the TMV 126/183 kDa proteins to carry out co-immunoprecipitation experiments on a replicase preparation from tobacco infected with TMV-OM. They found that anti-M precipitated both the 126 and 183 kDa proteins, with the 126 kDa protein being present in excess of the 183 kDa protein, while anti-P precipitated similar amounts of the 126 and 183 kDa proteins (Watanabe et al., 1999). This led them to suggest that the 183 kDa protein occurs in the viral replicase complex predominantly as a heterodimer with the 126 kDa protein. However, we have seen no interaction in the yeast two-hybrid system between either the Dom1 (methyltransferase) or Dom2 (helicase) constructs with the Dom3 (polymerase) construct (data not shown). This suggests that dimerization between the 126 and 183 kDa proteins requires either a bridging factor, such as a host factor, or involves an interaction between the polymerase domain of the 183 kDa protein and the region of the 126 kDa protein lying between amino acid residues 484 and 647 (Fig. 1).

In summary, we have used the yeast two-hybrid system to show that the yeast GCD10 protein appears to interact with the TMV 126/183 kDa proteins via the methyltransferase-like domain. Whether this represents a direct interaction between the two polypeptides or an indirect interaction via a third (bridging) factor present in yeast and plant cells awaits further studies in vitro. However, our work confirms and extends that of Osman & Buck (1997), who showed that a TMV replicase preparation purified from tomato contained a 56 kDa host protein related to the GCD10 subunit of yeast eIF-3. The interaction between a plant GCD10-like protein and the methyltransferase-like domain of the 126/183 kDa protein may constitute a link between the viral replicase and the host cell translation machinery. One possible function may be to facilitate transfer of nascent, capped (+)-strand RNA to the...
translation machinery of the host cell. Alternatively, the GCD10-like protein may have an as yet unidentified role in (−) strand and sub-genomic RNA synthesis. Our results suggest that synthesis of viral RNA may be closely coordinated with viral protein synthesis.

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References


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