SUMMARY

Many viruses that infect plants or animals are positive strand RNA viruses; they are classified in 20 families and 88 genera. Because the genes that are involved in replication are essentially conserved among viruses in the genera, these viruses can also be grouped into three large superfamilies or superfamilies, although these groupings have no taxonomic connotation. Given the basic similarities among members of each of these superfamilies, research data can be compared for understanding the basic mechanisms involved in virus genome replication and, in particular, how the host cell contributes to this process. The use of the budding yeast *Saccharomyces cerevisiae* was a breakthrough in these types of studies because systems were developed that have allowed replication in yeast cells of some (+)RNA plant (bromoviruses and tombusviruses) and animal (nodaviruses) viruses. Using yeast cells, many advances have been made in the comprehension of the virus-host interactions that lead to the formation of functional replication complexes and of the involvement in the process of several host genes.

*Key words*: positive strand RNA viruses, replication, host factors, yeast, *Saccharomyces cerevisiae*.

INTRODUCTION

Viruses are obligate parasites that invade cells, utilizing and altering the host cell machinery for their replication. They are responsible for a very large variety of human, animal and plant diseases that can cause severe economic losses. Virus genomes, which consist of one or more DNA or RNA segments, are relatively simple, which has allowed their encoded replication factors to be investigated extensively. However, the molecular processes involved in virus replication are complex, since these processes also involve a number of factors contributed by the host.

Viruses with single-stranded, positive sense messenger RNA [(+)-RNA] genomes are currently classified in 20 families and 88 genera (Fauquet *et al*., 2004). These viruses are also referred to as members of three large superfamilies or superfamilies based on the similarities among the virus-coded non-structural proteins that are essential for replication, i.e. RNA-dependent RNA polymerase (RdRp), and, if present, helicase and capping domains. Additional distinctive elements are related to the structures of the termini of genomic RNA, such as cap, VPg or a phosphate group at the 5’ end, poly(A) tails, tRNA-like structures or other higher order structures at the 3’ end (Koonin and Dolja, 1993).

Replication of (+)-RNA viruses invariably proceeds through the synthesis of negative-strand RNA molecules without DNA intermediates. Another major characteristic of these viruses is that they replicate in close association with host cell membranes, which proliferate and often rearrange in the form of vesicles, thus forming a sort of protected compartment in which virus replication-related non-structural proteins, template RNA and, probably, host factors accumulate for genome replication (Buck, 1996). Moreover, cell membranes sequester genomic RNAs preventing their engagement in translation and protect double-stranded replicating RNA from RNA interference or other host-triggered defence mechanisms. Different membranes can be specifically involved in the assembly of the replication complex. Depending on the virus, replication can be associated with endoplasmic reticulum (ER), nuclear envelope, mitochondria, peroxisomes, Golgi apparatus, lysosomes or endosomes. Targeting to the specific cellular compartment can be promoted by the RdRp itself or by other viral non-structural proteins. The basic similarities in the expression and replication strategies of (+)-RNA viruses encourage the analysis of simple viruses as models, because the information obtained could be of help for the comprehension of the mechanisms that underly the replication of other (+)-RNA viruses, including agents of important plant and human diseases. In particular, much attention is devoted to host factors involved in the replication of these viruses. The identification of host proteins that interact with virus-encoded proteins and viral RNA is important, because they may consti-
stitute a target for virus disease control. Furthermore, the characterization of host genes involved in virus replication could be of help to understand their role in cell biology. Various strategies are available for the identification of host factors involved in plant virus replication, which include purification and analysis of the replication complex from infected cells of natural hosts, screening for proteins able to bind virus RNA or virus-encoded proteins, and mutational analyses of a genetically simple plant host, such as *Arabidopsis thaliana* (Ahlquist et al., 2003, and references therein).

Genetic analysis for the identification of host factors involved in virus replication could be greatly facilitated if an organism simpler than *A. thaliana* could be infected with viruses that infect higher eukaryotes. The budding yeast *Saccharomyces cerevisiae* has been shown to be suitable for this purpose (Janda and Ahlquist, 1993).

Yeasts grow very fast in culture and thus have the advantages offered for decades by a bacterium such as *Escherichia coli* for the development of recombinant DNA technologies and molecular genetics. On the other hand, *S. cerevisiae* is a eukaryote, sharing therefore with higher organisms, like plants and animals, major properties, including the presence of organelles such as mitochondria, peroxisomes, endoplasmic reticulum (ER), Golgi apparatus, and nucleus with genetic material arranged in chromosomes.

Foreign genes can be introduced into yeast cells by using “shuttle vectors”, i.e. plasmids that can be propagated and amplified also in *E. coli*. Vectors that propagate extrachromosomally in yeast cells contain either a centromeric sequence (CEN) in addition to an autonomous replicating sequence (ARS) (plasmids YCp) or the replication origin of the yeast plasmid 2 µm (plasmids YEp). Vectors YCp propagate at low copy number and stably segregate in the progeny cells receiving one or two copies of the plasmid. Conversely, vectors YEp propagate at high copy number, usually 50-100 copies per cell. However, they are not stable and segregate irregularly leading to some copy number variation within the progeny. As an alternative to using autonomously propagating plasmids, foreign DNA could be integrated into the yeast genome by homologous recombination. Vectors for this purpose are called YIp (Parent and Bostian, 1995).

Yeast vectors contain a multiple cloning site region and encode selectable markers that can complement specific auxotrophies, to allow selection of transformants. Furthermore, vectors contain yeast transcription promoter and terminator sequences, upstream and downstream, respectively, of the polylinker region. Promoters can be either inducible (for instance, GAL1, inducible by galactose, CUP1, inducible by copper, or CTA, inducible by oleic acid) or constitutive (for instance, ADH1).

Methods for yeast transformation are diverse. In the most popular, cells are made competent by treatment with lithium acetate, and recombinant DNA is introduced into competent cells by heat shock. Methods for growth, transformation, and selection of transformants are extensively described in manuals (e.g., Ausubel et al., 1989).

### POSITIVE STRAND RNA VIRUS GENE EXPRESSION AND REPLICATION

The pioneer work of P. Ahlquist and co-workers in the use of the genetically tractable yeast *S. cerevisiae* as a model to study positive strand RNA virus replication mechanisms has opened up new virological perspectives. Following the first reports on the replication of *Brome mosaic virus* (BMV) in yeast cells (Janda and Ahlquist, 1993), several other viruses with RNA or DNA genomes have been shown to replicate partially or fully in this host (Price et al., 1996; Angeletti et al., 2002; Zhao and Frazer, 2002; Pantaleo et al., 2003; Panavas and Nagy, 2003; Raghavan et al., 2004; Price et al., 2005).

This article will focus mainly on (+)RNA virus-encoded replication factors involved in virus replication, i.e., the expression of the RdRp and other viral products responsible for the intracellular localization of the virus replication complex. An overview will also be given of the host factors that have been identified so far as being involved in virus replication in yeast (see also Noueiry and Ahlquist, 2003; Ahlquist et al., 2003; Ahlquist et al., 2005).

**Brome mosaic virus.** *Brome mosaic virus* (BMV) is the type species of the genus *Bromovirus* (family *Bromoviridae*) in the alphavirus-like superfamily (or supergroup III) (Ahlquist, 1992). The genome, constituted by three RNAs (RNA1, RNA2, RNA3), is capped at the 5’end and can fold into a tRNA-like structure at the 3’end. RNA1 (3.2 kb) and RNA2 (2.9 kb) encode the replication proteins, 1a and 2a, respectively. The 109-kd 1a protein contains an N-terminal capping and a C-terminal helicase domain. Protein 2a (94 kDa) contains motifs characteristic of RdRps. RNA3 (2.1 kb) encodes the 32-kDa cell-to-cell movement protein (protein 3a) in the 5’ region and the 20-kDa coat protein (CP) in the 3’ region. After replication of RNA3, the CP is translated from a subgenomic RNA (RNA4) that is synthesized from negative-strand RNA3.

Proteins 1a and 2a have been constitutively expressed in yeast from plasmids, whereas wild type (wt) or RNA3 derivatives were introduced into these cells either by transfection of *in vitro* synthesized RNA (Janda and Ahlquist, 1993) or by *in vivo* transcription from plasmids under the control of the GAL1 promoter (Ishikawa et al., 1997a). The replication of BMV RNA3 was ascertained by the synthesis of subgenomic RNA4.
from negative-strand RNA3. The expression of reporter genes replacing the 3' proximal CP gene in RNA3 was a further proof of RNA3 replication in yeast cells, e.g. expression of a URA3 gene that allowed the growth of ura3 yeast cells in a medium lacking uracil. The yeast system was used to study the relationships between RNA3 replication and subgenomic RNA4 transcription, and it was shown that the two processes inhibit each other because of competition for the same negative-strand RNA3 template (Grdzelishvili et al., 2005).

Analysis of the intracellular distribution of proteins 1a and 2a, and of the RNA progeny showed that they localize to perinuclear ER (Restrepo-Hartwig and Ahlquist, 1999), which is similar to their localization in infected plant cells (Restrepo-Hartwig and Ahlquist, 1996). Further cytological analysis with the electron microscope showed that the sites of accumulation of replication proteins and virus RNA are constituted by perinuclear masses of vesicles or spherules, 50-70 nm in diameter, which contain fine fibrils, probably consisting of RNA. In several cases, the spherules were connected to the outer nuclear membrane, appearing as invaginations with a neck opening to the cytoplasm (Schwartz et al., 2002). These cytological features parallel those observed in plant cells infected by members of the family Bromoviridae (see for review Martelli and Russo, 1985).

Combined biochemical and immunomicroscopic analyses showed that the formation of vesicles can be induced by the sole presence of protein 1a. When 1a is co-expressed with protein 2a and RNA3, the vesicles become the site of virus RNA replication (Schwartz et al., 2002). Interestingly, modulation of the relative expression of proteins 1a and 2a (by using appropriate inducible promoters) changed the site of virus replication from perinuclear vesicles to stacked, double-membrane layers, thus showing that the association with an organelar membrane for a given virus is not strictly related to specific host factors supplied uniquely by that membrane (Schwartz et al., 2004).

The expression separately of the components of the BMV replication complex in yeast made it possible to show that protein 1a localizes to the ER independently of 2a and template RNA (Restrepo-Hartwig and Ahlquist, 1999) and that the association with the ER membrane is mediated by sequences in the N-terminal RNA capping domain (de Boon et al., 2001). On the contrary, targeting and localization of 2a to ER requires interaction with protein 1a (Chen and Ahlquist, 2000). In particular, by using a series of deletion mutants of protein 2a, it was shown that the interaction of 2a with 1a was due to sequences near the 2a N-terminus. In the absence of protein 1a, 2a is not associated with any cell organelle, and remains in the cytoplasm in a diffused form or in small aggregates (Chen and Ahlquist, 2000).

Protein 1a also had a key role in the formation of the BMV replication complex and in the stabilization and recruitment of the RNA3 template to the replication site (Janda and Ahlquist, 1998). This finding was again made possible by expressing separately proteins 1a and 2a in cells together with BMV RNA3 transcripts. It was shown that in the presence of protein 1a alone, replication and translation of RNA3 are blocked so that this RNA is protected from degradation by the exonuclease activity that normally follows translation, and is transferred to the replication site in close association with cell membranes (Janda and Ahlquist, 1998; Schwartz et al., 2002). It was also shown that the formation of an active replication complex requires the simultaneous expression of the replication proteins and template RNA (Quadt et al., 1995). Further analysis showed that the 1a-mediated RNA3 stabilization depends on sequences contained in the intergenic replication enhancer region [IRE, a sequence between the 3a and CP genes (Sullivan and Ahlquist, 1999)] and on both the capping and helicase domains of protein 1a (Ahola et al., 2000). As it does for RNA3, protein 1a also stabilizes BMV RNA2. This RNA can also be replicated in yeasts by supplying protein 1a in trans and protein 2a either in trans or in cis (Chen et al., 2001). In both cases, RNA2 is stabilized by protein 1a and is targeted to membranes. Sequences essential for the reactivity of RNA2 were mapped in the 5' untranslated region and the N-terminal region of ORF 2a (Chen et al., 2001).

As mentioned above, one major aim of using S. cerevisiae as an alternative host for viruses of higher eukaryotes is the identification of host genes that participate in virus replication. Information from the fully sequenced S. cerevisiae genome was extensively used for the analysis of host genes required for BMV genome expression and RNA replication. This showed the involvement of a number of host factors in this process (Ishikawa et al., 1997b; Diez et al., 2000; Lee et al., 2001; Noueiry et al., 2000, 2003; Noueiry and Ahlquist, 2003).

The protein complex Lsm 1p-7p/Pat1p, encoded by the LSM1 to LSM7 and PAT1 yeast genes that are involved in deadenylation-dependent mRNA decapping, regulates switching of the replicable RNA1, RNA2, and RNA3, but not of the non-replicable RNA4 or other cellular mRNAs, from the translation machinery to the replication site. In mutant yeast strains that do not express this protein complex, BMV RNA translation was inhibited because ribosome scanning of the viral RNA reading frame was slowed down or stalled, so that the RNA could not be recruited by the protein 1a (Diez et al., 2000; Noueiry et al., 2003).

DED1 is another host gene directly involved in translating BMV mRNAs (Noueiry et al., 2000). It encodes an RNA helicase whose action is essential for translation initiation. In the mutant allele, only translation of BMV RNA2, which encodes the RdRp protein 2a, was selectively inhibited. The translation of RNA1 and that of cellular mRNAs was unaffected, and the functionality of
protein 1a was maintained. Specific inhibition of RNA2 translation was due to a short sequence present in the 3' untranslated region of this RNA, but not in RNA1. The dependence of RNA2 translation on the activity of the DED1 cellular gene suggests that it is a means to down-regulate the synthesis of protein 2a in relation to protein 1a, a mechanism analogous to down regulation by translational read-through or frameshift in cells infected by monopartite RNA genome viruses.

The synthesis of minus-strand BMV RNA is blocked in yeast cells bearing mutation in the YDJ gene (Tomita et al., 2003). This gene encodes a protein, Ydj1p that is a part of the chaperone complex involving HSP90 and HSP70. It was shown that proteins 1a and 2a, and template RNA were associated with ER as in wild type cells. However, in these cells, RNA3 negative strand synthesis was inhibited and a large portion of protein 2a was aggregated in the cytosol rather than being associated with membranes. These results suggested the involvement of Ydj1p in the activation of the replication complex for the synthesis of negative-strand RNA perhaps by interacting with the correct folding or assembly of protein 2a in the replication complex.

The formation of spherular vesicles in the perinuclear ER, where protein 1a, protein 2a and template RNA are found, and where RNA synthesis takes place, depends on the correct ratio between unsaturated (UFA) and saturated (SFA) fatty acids. The synthesis of UFA is catalyzed by an enzyme, D9FA desaturase (Ole1p), which converts SFA into UFA. Ole1p is encoded by the gene OLE1. Mutation in this gene inhibits negative-strand BMV RNA synthesis without altering cell growth (Lee et al., 2001; Lee and Ahlquist, 2003). The inhibition of virus RNA synthesis was probably due to a decrease in the fluidity of the membrane of the perinuclear spherules, which seems to be necessary for the proper interactions of proteins 1a and 2a with each other or with host factors. The specific sensitivity of BMV RNA replication to lipid composition of host cells prompted Lee and coworkers to suggest that altering the ratio UFA/SFA in host cells could be the basis for a useful antiviral strategy (Lee et al., 2001; Lee and Ahlquist, 2003).

The mutational analyses summarized above were conducted on yeast cells exposed to UV light or ethyl methanesulphonate and screened for inhibition of BMV-directed expression of reporter genes. To secure quicker means for identification of host genes that influence BMV replication in yeasts, about 4,500 deletion S. cerevisiae strains, encompassing almost 80% of the yeast genome, were analyzed (Kushner et al., 2003). About 100 genes were identified that inhibited or stimulated BMV RNA replication. Several were the same as those identified by using the traditional analyses reported above. Others were found to influence one or another step of BMV RNA replication, whereas the function of some other genes was not elucidated.

The authors stressed the need for further studies to characterize the involvement of these host factors in BMV RNA replication. Microarray technology can be of assistance in these studies, because the analysis of differential gene expression in different cellular states may be of help for the identification of up- and down-regulated cell genes during virus replication. In addition, the comparison between wild type and mutant yeast strains may shed light on gene functions. A semiparametric hierarchical mixture method has been shown to be an appropriate bioinformatic approach to obtain a rank ordering of genes (Newton et al., 2004).

**Genus Tombusvirus.** Species of the genus *Tombusvirus* (family *Tombusviridae*) have the typical genome of the superfamily of carmo-like viruses (supergroup II) in that they encode an RdRp protein, but have neither a capping or helicase domains, nor a proteinase. Their monopartite genome is a single-stranded RNA of ca. 4.8 kb, lacks a 5' cap structure and is not polyadenylated. It contains five ORFs. The 5'-most ORF encodes a protein of 36- or 33-kDa (p36 or p33), whereas ORF 2 encodes a 95- or 92-kDa protein (p95 or p92), which is expressed by readthrough of the amber stop codon of ORF 1. The polymerase domain is contained in the readthrough region of ORF 2; however, both ORFs 1 and 2 must be expressed for the replication of virus RNA. ORF 3 codes for the CP, and two nested ORFs, 4 and 5, encode two proteins (21-kDa and 19-kDa) that are involved in virus movement and symptom expression in infected plants, respectively (Russo et al., 1994). Moreover, the 19-kDa protein has been identified as a suppressor of post transcriptional gene silencing (PTGS) (Voinnet et al., 1999; Silhavy et al., 2002). The simplicity of the tombusvirus genome has made these viruses ideal models for the study of (+)RNA virus replication. ORFs 3, 4 and 5 are dispensable for replication in protoplasts, in which only the expression of ORFs 1 and 2 is required. Infection of tombusviruses is normally associated with small defective-interfering (DI) RNAs, i.e. deletion mutants of the viral genome. Since DI RNAs do not code for any protein and depend exclusively on the viral helper genome for replication (Russo et al., 1994), they represent an additional useful model for studying virus and host replication factors.

The yeast system was used to characterize the relation of the viral replicase with host membranes and to study replication itself. Circumstantial evidence from cytopathological studies had suggested that tombusvirus replication takes place in vesiculated structures (multivesicular bodies) derived from proliferation of the outer membrane of mitochondria in the case of *Carnation Italian ringspot virus* (CIRV) and for Pelargonium necrotic spot virus (PNSV) (Heinze et al., 2004), or from the peroxisomal membrane for *Cymbidium ringspot virus*.
(CymRSV) and all other members of the genus (Russo et al., 1987). The determinants for the origin of the vesicles, whether from mitochondria or peroxisomes are contained in the N-terminal region of ORF 1 (Burgyan et al., 1996). Expression of GFP fusions of CIRV ORF 1 gene product (p36) in yeast cells showed that GFP fluorescence was localized to structures that could be identified as mitochondria. Contrary to healthy controls (Fig. 1), electron microscope observations of cells expressing p36 fused or not to GFP showed an increased number of morphologically modified mitochondria, forming large aggregates together with membranous elements, that were often apposed to mitochondria and arranged in ordered stacks (Rubino et al., 2000) (Fig. 2). Immunoelectron microscopy confirmed the association of p36 with the membrane aggregates (Rubino et al., 2000). Also, in yeast cells, CIRV p36 was anchored to the mitochondrial outer membrane with both N- and C-termini on the cytoplasmic side by means of 113 amino acids containing two transmembrane domains. Targeting to the mitochondrial outer membrane was mediated by the same two transmembrane segments plus multiple recognition signals in the N-terminal region of ORF 1 (Weber-Lotfi et al., 2002).

Fig. 1. Transection of a control S. cerevisiae cell. N = nucleus, M = mitochondria. Bar = 300 nm.
Similar analyses of yeast cells expressing the ORF 1-encoded protein of CymRSV (p33) showed that this protein was targeted to the peroxisomal membrane, where it was anchored, with both N- and C-termini free in the cytoplasm, through a sequence of ca. 70 amino acids containing two transmembrane domains. The targeting signal was identified in this sequence plus a basic tripeptide (KRR) upstream of the transmembrane segments. Expression of p33 in S. cerevisiae cells induced the formation of peroxisomal aggregates intermingled with membranes (Navarro et al., 2004).

Since these studies had shown that targeting and anchoring properties of the replicase proteins of tombusviruses to mitochondrial and peroxisomal membranes of plant cells are maintained in yeast cells, these cells were also tested as possible hosts for the replication of tombusvirus RNA. The proteins encoded by CIRV ORFs 1 and 2 were constitutively expressed through the ADH1 promoter, whereas a DI RNA molecule was expressed through the galactose-inducible GAL1 promoter and terminated near its natural 3'end by a ribozyme derived from Tobacco ringspot virus satellite RNA (Pantaleo et al., 2003). CIRV replicase-dependent amplification of DI RNA was measured by using several parameters including incorporation of 5'-bromo-UTP in the progeny and the synthesis of negative strands (Pantaleo et al., 2003; Rubino et al., 2004). The suitability of yeast cells to replicate DI RNAs was confirmed by using replicase proteins derived from CymRSV (B. Navarro, L. Rubino, V. Pantaleo and M. Russo, unpublished results) and Cucumber necrosis virus (CNV; Panavas and Nagy, 2003). An active template-replicase protein complex was purified from yeast cells that had expressed the CNV replicase proteins and DI RNA (Panaviene et al., 2004).

The yeast system was further explored to analyse the interaction between CIRV replicase proteins p36 and p95, template DI RNA, and host membranes (Pantaleo et al., 2004). It was shown that both proteins target independently to mitochondrial membranes to which template and progeny RNA are also bound. In the absence of both proteins, DNA-directed DI RNA transcripts were rapidly degraded, whereas in the presence of either p36 or p95, a small fraction of the DI RNA was protected from degradation and became associated with membranes.

To confirm the stabilizing effect of replicase proteins on DI RNA transcripts, a non-replicable RNA was co-expressed with one or both proteins. It was found that a

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Fig. 2. Transection of a S. cerevisiae cell transformed with a plasmid expressing CIRV p36, showing deranged mitochondria (M) and extensive membrane proliferation. N = nucleus. Bar = 300 nm.
small portion of DI RNA transcripts was stabilized and targeted to membranes when p36 or p95 were expressed. By contrast, when both proteins were co-expressed, a larger amount of DI RNA was targeted to membranes. By modulating the expression of one or both proteins, using the copper-inducible CUP1 promoter, a higher level of p36 than p95 was required for DI RNA replication in yeast, because p36 concentration must reach a threshold level to stabilize DI RNA efficiently. This is in line with the results of in vitro studies that showed the co-operative “all-or-none” behaviour of tombusvirus ORF1 protein (Rajendran and Nagy, 2003).

Comparing the results of these studies with those on BMV revealed an important difference between the two systems. Whereas with BMV a single protein (1a) is responsible for recruiting the other replicase protein and template RNA, with CIRV, either p36 or p95 can target themselves and DI RNA to membranes independently; however, both are required for the efficient stabilization and targeting of template RNA to the replication site.

The suitability of the yeast system to study tombusvirus replication was further tested with virus genomic RNA, either as a complete functional molecule (unpublished information) or as deletion mutants containing only ORFs 1 and 2, that were able to replicate in plant cells (Rubino et al., 2004). No replication was detected, at least under the conditions of the experiments. Moreover, the lack of, or poor, replication of genomic RNA was confirmed by the fact that no replication was detected of a satellite RNA (Rubino et al., 1990) added to the system. For satellite replication, replicase proteins must be supplied by a cis replicating genome (Rubino et al., 2004). Efforts to ameliorate the yeast system to allow the synthesis of genomic RNA are under way.

A systematic analysis of S. cerevisiae genome recently carried out for identifying host genes involved in tombusvirus replication showed that 96 different genes of the yeast affected the replication of Tomato bushy stunt virus DI RNA (Panavas et al., 2005).

**Genus Alphanodavirus.** Flock House virus (FHV) is an insect virus of the genus Alphanodavirus (family Nodaviridae) that shares genome properties with viruses of the alphavirus-like superfamily (Ball and Johnson, 1998). The 4.5-kb genome of FHV is bipartite, consisting of two uncapped, non-polyadenylated RNAs (RNA1 and 2), packaged in a single icosahedral virion. RNA1 (3,100 nt) encodes a protein (protein A) of 112-kDa, which is the viral RdRp; RNA2 (1,400 nt) codes for the CP. RNA1 can replicate autonomously in the absence of RNA2, whereas RNA2 can replicate only by using the replicase translated from RNA1. A subgenomic RNA of 400 nt (RNA3) is transcribed from RNA1 during replication. This RNA acts as mRNA for the synthesis of two small proteins, B1 and B2. The role of protein B1, which is dispensable for FHV replication, is unknown, whereas the function of protein B2 is to maintain RNA1 replication upon repeated serial RNA passages. RNA3 replication is essential to trans-activate that of RNA2.

A peculiar characteristic of FHV is that its RNA can infect insect, plant and mammalian cells (Ball and Johnson, 1998), suggesting that host components involved in virus replication are highly conserved. This prompted investigation of the ability of S. cerevisiae to support FHV replication.

Yeast cells were either transfected with phenol-extracted virus RNAs 1 and 2 or were transformed with a plasmid from which RNA1 could be transcribed (Price et al., 1996). Transfection with genomic RNAs 1 and 2 led to efficient replication of both these RNAs including synthesis of the viral CP and the assembly of virus particles. Furthermore, subgenomic RNA3 was correctly transcribed from RNA1. Yeast cells expressing RNA1 from a 2 µm plasmid also allowed replication of RNA1 and transcription of RNA3. Interestingly, no specific yeast promoter was included in this plasmid, which was only engineered to produce the exact viral 3’ end by linking the 3’ end of the RNA1 cDNA to the Hepatitis delta virus (HDV) ribozyme cDNA. RNA1 transcription was controlled by an unknown cryptic promoter upstream of the 5’ terminus of viral RNA. When yeast cells were transfected also with an RNA2 derivative, in which the CP-coding sequence was replaced by the URA3 gene, prototrophs could be selected that grew in the absence of uracil for many generations after transfection.

The FHV-yeast system was further refined by using a plasmid that contained the yeast GAL1 promoter next to the 5’ end of RNA1 (Price et al., 2000). In yeast cells containing this plasmid, RNA1 molecules with natural 5’ ends were selectively amplified and RNA1 accumulation was comparable to that in infected Drosophila cells (the standard host for FHV propagation). In contrast, the level of RNA3 was higher in yeast than in insect cells, in line with the notion that the replication of RNA2 (absent in this yeast system) inhibits RNA3 synthesis (Zhong and Rueckert, 1993).

Insertion of the green fluorescent protein (GFP) reporter gene in frame with, or after, protein A, or in subgenomic RNA3 allowed the expression of the foreign gene and the direct visualization of cells sustaining RNA1 or subgenomic RNA3 replication. However, the result was affected by the site of GFP insertion (Price et al., 2000). In other experiments, chimeric RNA2 containing either the URA3 or HIS3 gene and the 5’ and 3’ terminal sequences of RNA2 was transcribed in vivo from plasmids under the control of the GAL1 promoter. The constructs were such that the reporter genes could only be expressed upon replication of RNA1, allowing selection on an appropriate growth medium (Price et al., 2002). By using yeast clones in which the RNA1 template containing a protein A frameshift was replicated in trans by coexpression of functional protein
A from a separate unreplicable RNA1 derivative, cis sequences required for RNA1 replication and subgenomic RNA3 synthesis were identified (Lindenbach et al., 2002). Two RNA1 cis replication elements (RE) were mapped internally (intRE) and contiguous to the 3’ terminus (3’RE) of RNA1. In addition, two subgenomic control elements, proximal (PSCE) and distal (DSCE) to the transcription start site of RNA3, were identified in the FHV RNA1 sequence, acting via long-distance base pairing in RNA1. It was suggested that these findings could be useful for the construction of FHV RNA3-based expression vectors. It was also shown that, besides controlling subgenomic RNA3 synthesis, base-pairing between PSCE and DSCE was essential for trans replication of RNA2 (Lindenbach et al., 2002).

FHV-infected Drosophila cells exhibit typical mitochondrial aggregation and the formation of spherical vesicles consisting in invaginations of the mitochondrial outer membrane in the intermembrane space, where virus RNA replication is thought to take place (Miller et al., 2001). As shown by biochemical and microscopic analysis of yeast cells, the FHV replication complex is anchored to the outer mitochondrial membrane by the RNA1-encoded protein A via a targeting signal and a transmembrane domain present in the N-proximal region, with the rest of the protein exposed to the cytoplasm (Miller and Ahlquist, 2002). Targeting and anchoring properties of protein A do not depend on any other virus-encoded factors or on RNA1 replication and subgenomic RNA production. The N-terminal 46 amino acids of protein A are sufficient to target and anchor GFP to mitochondria (Miller and Ahlquist, 2002). Vesicles reminiscent of those found in infected insect cells are also found in yeast cells that support RNA1 replication, although they are much less conspicuous. The protein A outer membrane mitochondrial signal could be substituted for by an ER targeting signal without affecting replication. The ultrastructural appearance of yeast cells expressing the ER-targeted protein A showed that wild-type protein A-induced clusters of membrane-bounded mitochondria, with no cristae, were substituted for by perinuclear membrane proliferation, with multiple membrane layers apposed to the nucleus. In this case, it was shown that specific cell membranes are not strictly required for a given (+)RNA virus (Miller et al., 2003).

Nodamura virus (NoV), the type member of genus Alphanodavirus, has a bipartite genomic organization similar to that of FHV. Complete replication of NoV was obtained in S. cerevisiae cells, either after transfection with viral RNA or by DNA-dependent in vivo transcription of virus genome (Price et al., 2005). As reported for FHV (Price et al., 2002), NoV RNA2-based replicons supported by RNA1 replication could be used for the expression of heterologous genes to select replication-dependent colonies. The requirement for subgenomic RNA3 replication to transactivate RNA2 replication (Eckerle and Ball, 2002) was also confirmed for NoV (Price et al., 2005).

Nodaviruses are so far the only (+)RNA viruses whose complete replication has been obtained in yeast cells. RNA replication in the heterologous system conformed to the virus replication cycle in the natural host cells, including the synthesis of subgenomic RNA3 and the formation of mature virions (Price et al., 1996). In addition, infected yeast cells contained virus-induced cyto logical alterations that mirrored those found in Drosophila cells, i.e. mitochondrial clustering and invagination of the organelle outer membrane to form spherules.

CONCLUDING REMARKS

S. cerevisiae has been shown to be a powerful tool for studying the interactions between positive-strand RNA viruses and their host cells. The results obtained so far have given an insight into the assembly and functions of the viral replication complex in the context of this fully characterized yeast. As a result, important conclusions have been reached as to the host factors that are involved in the replication processes. This information casts light on the role of these genes in normal cellular pathways that so far have not been characterized.

The knowledge of viral and host factors involved in virus replication can be of great importance for the development of specific antiviral strategies. In addition, following the thorough characterization of the mechanisms underlying (+)RNA virus replication, it is conceivable that these viruses could be used as vectors for the controlled expression of foreign sequences for the production of, for instance, antigens. The extension of studies in S. cerevisiae to the fission yeast Schizosaccharomyces pombe will strengthen the validity of this model system, also allowing the study of PTGS in virus infections, because this species, unlike S. cerevisiae, contains all the components of the PTGS system (Aravind et al., 2000; Sigova et al., 2004).

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