Coupling of killer virus transcription with translation in yeast cell-free extracts

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The cytoplasmically inherited killer virus of *Saccharomyces cerevisiae* expresses its dsRNA genome via apparently uncapped viral transcripts produced in the cytoplasm of infected cells. Virions of this naturally temperature-sensitive virus can be added to cell-free translational extracts of uninfected yeast cells resulting in a reaction in which viral transcription and translation are coupled at 15 °C in vitro. In this reaction nucleotides are incorporated into full-length transcripts of the M and L-A dsRNA segments, with lower levels of incorporation into genomic RNA. In addition, incorporation of nucleotides is observed into a smaller RNA species showing no sequence relatedness to M or L-A.

The killer virus of *Saccharomyces cerevisiae* is a cytoplasmically inherited virus the genome of which consists of two linear segments of dsRNA, denoted L-A dsRNA (4.9 kbp) and M dsRNA (1.8 kbp). M dsRNA is best regarded as a satellite RNA, since it depends on L-A for its replication (reviewed by Tipper & Bostian, 1984; Wickner, 1986). Killer yeast strains harbour virus like particles (VLPs) containing both genomic segments, which are separately encapsidated. These doubly infected strains produce a toxin which kills yeast strains lacking M dsRNA. Killer strains are resistant to toxin. Non-killer yeast strains generally contain L-A dsRNA, but some lack both dsRNA segments of this virus. L-A dsRNA encodes the major capsid protein of the VLPs (Hopper *et al.*, 1977) which is the product of an open reading frame encoding a polypeptide with an estimated *Mr* of 76K (Diamond *et al.*, 1989; Icho & Wickner, 1989), and has been observed to be 76K to 88K (Diamond *et al.*, 1989; Hopper *et al.*, 1977; Icho & Wickner, 1989; Thiele *et al.*, 1984). It is referred to here as 81K since its electrophoretic mobility on SDS–polyacrylamide gels corresponds to this size. In addition, L-A encodes a larger 180K polypeptide believed to be a capsid protein–dsRNA polymerase fusion generated by a –1 frameshift within the 129 bases preceding the capsid UAA termination codon (Diamond *et al.*, 1989; Fujimura & Wickner, 1988a; Icho & Wickner, 1989). The toxin and resistance factor are processed products of a preprotoxin polypeptide (estimated to be 32K to 36K) encoded by M dsRNA (Bostian *et al.*, 1980; Lolle *et al.*, 1984).

Killer VLPs isolated from yeast contain DNA-independent RNA polymerase activity which catalyses the synthesis of full-length positive-polarity transcripts of the M and L-A dsRNA molecules encapsidated in the VLPs in vitro (Bruenn *et al.*, 1980; Hannig *et al.*, 1984; Thiele *et al.*, 1984; Welsh & Leibowitz, 1980). The m and l transcripts of M and L-A dsRNA are released from the VLPs (Welsh *et al.*, 1980), and can be used as templates for in vitro translation by a yeast cell-free extract (Hussain & Leibowitz, 1986).

In prokaryotes, where transcription and translation of cellular and viral genes occur in the same cytosolic compartment, transcription and translation are known to be coupled in vivo, i.e. translation initiates on nascent mRNA molecules before transcription has terminated (Miller *et al.*, 1970). This coupling can be reproduced in vitro in a DNA-dependent coupled system (Chen & Zubay, 1983). In eukaryotic cells, nuclear transcription and cytoplasmic translation of cellular genes are not coupled, and occur in distinct subcellular locations. On the other hand, the transcripts of cytoplasmic viruses of eukaryotes are translated in the same cellular compartment in which they are synthesized, raising the possibility that transcription and translation might be coupled in vivo. We have developed a system for coupling killer virus transcription and translation in vitro in yeast cell-free extracts. Similar coupled systems have been established for vaccinia virus (Pelham *et al.*, 1978) and vesicular stomatitis virus (Ball & White, 1976).

Cell-free translation extracts were prepared (Hussain & Leibowitz, 1986; Leibowitz *et al.*, 1991) from logarithmically growing cells of *S. cerevisiae*. Extracts were titrated with micrococcal nuclease (Worthington) to determine the optimal treatment which would destroy

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endogenous mRNA in each extract. Extracts were prepared from strains M793 (a ura1 his7 lys2 met5 leu2-1 kex2-2 [KIL-O]) and M816 (a ade1 ade2 ura1 tyr1 his7 lys2 gal1 mkr1 KIL-O)), which are both heat-cured (Wickner, 1974) derivatives of killer strains. Neither strain contains M dsRNA; M816 also lacks L-A dsRNA but contains L-BC dsRNA, the 4.9 kbp genome of an unrelated virus (Thiele et al., 1984), and M793 contains both L-A and L-BC dsRNA.

Killer VLPs containing M and L-A dsRNA (with contaminating L-BC) were purified from stationary phase ethanol-grown cells of the diploid strain A364A × S7 as described (Thiele et al., 1984), and were used to catalyse viral transcription in vitro (Welsh & Leibowitz, 1980). Following transcription, m transcript, which contains an internal polyadenylate tract, was purified by oligo(dT)–cellulose chromatography (Hannig et al., 1984), and l transcript was purified by agarose gel electrophoresis.

Coupled transcription–translation reactions were performed at 15 °C (unless otherwise noted) in 25 μl reaction mixtures in the presence of 50 μM of each of 19 non-radioactive amino acids (omitting methionine), 56 mM-HEPES–KOH pH 7.4, 5 mM-Tris–HCl pH 7.4, 0.5 mM-MgSO4, 14 mM-NaCl, 60 mM-potassium acetate, 40 mM-KCl, 4.5 mM-DTT, 0.5 mM each of ATP, CTP, GTP, UTP, 20 mM-creatine phosphate, 0.5 mM-spermidine, 0.5 mM-putrescine, 2 mM-glucose-6-phosphate, yeast tRNA (40 μg/ml), creatine phosphokinase (20 μg/ml; Sigma), RNasin (200 units/ml; Promega), 2% glycerol, micrococcal nuclease-treated translation extract (100 to 125 μg protein), VLPs containing approximately 12.5 μg of viral protein, determined as described (Groves et al., 1968). These reaction conditions include components contributed by cell-free extracts and VLP preparations. For analysis of protein products, the reactions included L-[35S]methionine (12 to 18 μCi/reaction, translation grade, > 800 Ci/mmol; New England Nuclear). For analysis of RNA products, 50 μM-L-methionine was included and [α-32P]UTP (5 to 8 μCi/reaction, 3000 Ci/mmol; New England Nuclear) was added in addition to non-radioactive 0.5 mM-UTP.

The temperature optimum for killer virus-associated RNA polymerase activity (transcriptase) is approximately 28 °C (Georgopoulos & Leibowitz, 1984) whereas the yeast cell-free transcription reaction is optimal at 20 °C (I. Hussain, T. L. Williams & M. J. Leibowitz, unpublished results). When translation was performed at 25 °C, 35S-labelled protein products appeared to be degraded within 120 min; this degradation was not observed at 20 °C (data not shown). The temperature optimum for coupled transcription and translation, as measured by incorporation of [35S]methionine into TCA-precipitable material or into appropriate polypeptides on SDS-PAGE (12.5% acrylamide) was found to be 15 °C, lower than for either reaction alone (Fig. 1). Although 3 h reactions did not show a significant difference between reactions run at 12 °C and 15 °C, shorter reaction times consistently showed the rate of methionine incorporation to be optimal at 15 °C (data not shown). The 15 °C temperature optimum of the coupled system is significantly below the optimum for viral transcription (28 °C) or translation in vitro (20 °C). This observation may relate to the natural temperature sensitivity of most strains of the virus, which is cured by growth of infected cells at elevated temperature (37 °C). Curability varies among different virus-infected strains (Wickner, 1974; Zorg et al., 1988). The molecular basis for curability is unknown; development of an in vitro replication system may allow the temperature-sensitive component to be identified.

Purified killer VLPs contain a mixture of L-A dsRNA- and m dsRNA-containing particles with variable but lower levels of L-BC dsRNA particle contamination (Thiele et al., 1984). When the translational extract was prepared from strain M816, production of both 81K capsid and 32K preprotoxin polypeptides was dependent on addition of VLPs containing these dsRNA species (Fig. 2a). On the other hand, in extracts of strains such as M793, which contain endogenous L-A dsRNA-containing transcriptionally active VLPs, production of the 81K polypeptide was not totally dependent on the addition of exogenous VLPs (Fig. 2b). In the absence of added VLPs, extracts of strain M816, which contains endogenous L-BC dsRNA-containing virions, synthesize a 77K polypeptide (Fig. 2a, lanes 8 to 10) which corresponds to the major capsid protein of this class of VLPs (Thiele et al., 1984). The identity of the protein bands below 30K seen in the coupled reactions in Fig. 2 is unknown. The 32K product of the coupled system reaction was shown to...
Fig. 2. SDS-PAGE of 35S-labelled protein products from the coupled system. (a) Cell-free extracts from strain M816 were incubated under coupled transcription-translation conditions in the presence of A364A x S7 VLPs for 0, 30, 60, 120, 180, 300 and 420 min (lanes 1 to 7), and in the absence of added VLPs for 120, 180 and 300 min (lanes 8 to 10). Lane 11 represents the products translated in a reaction containing purified m viral transcript instead of added VLPs. The band denoted P is the 32K preprotoxin protein. The 81K major capsid protein (C) is the upper band of the doublet in the indicated region of the gel. The mobilities of Mr standards are indicated (lane 12). (b) Cell-free extracts derived from strain M793 were incubated under coupled system conditions in the presence of added VLPs and the absence of UTP for 0 min (lane 1), 30 min (lane 2), 60 min (lane 3), 120 min (lane 4), 180 min (lane 5), 300 min (lane 6) or 420 min (lane 7). Lanes 8 (180 min) and 9 (300 min) are reactions run in the absence of added VLPs and in the presence of UTP. Reactions in the presence of both added VLPs and UTP are shown in lanes 10 (180 min) and 11 (300 min). Lane 12 contains Mr standards. In lanes 8 to 11 the high Mr product may represent the predicted 180K capsid–polymerase fusion protein.

be preprotoxin by immune precipitation with anti-toxin antibody as previously described (Hussain & Leibowitz, 1986).

When cell-free extracts of strain M793, which contains endogenous L-A and L-BC dsRNA, were incubated in the complete coupled system with added VLPs, both capsid (C) and preprotoxin (P) proteins were produced (Fig. 2b, lanes 10 and 11). The omission of added UTP virtually eliminated synthesis of both proteins (lanes 1 to 7). If the complete coupled system reaction was run without added VLPs, capsid protein and the protein comigrating with the capsid protein of endogenous L-BC dsRNA-containing VLPs were produced, but no preprotoxin was synthesized (lanes 8 and 9). Thus, in vitro synthesis of the protein products of viral dsRNA segments requires the presence of VLPs and UTP. Since UTP is not required for cell-free translation, transcription of the viral dsRNA molecules appears to be required for subsequent translation, indicating that both processes are occurring in the coupled system. The small quantity of preprotoxin produced after long incubations in the absence of added UTP (Fig. 2b, lanes 6 and 7) may reflect the presence of small quantities of UTP or UDP in the VLP preparation or extract, despite extensive dialysis of the VLPs and removal of nucleotides from the extract by Sephadex G-10 chromatography. UDP could be converted to UTP by VLP-associated nucleoside diphosphate kinase activity (Georgopoulos & Leibowitz, 1987). Alternatively, this UTP-independent translation could be due to disassembly of a minor particle fraction containing positive-stranded RNA (Fujimura et al., 1986) providing translational template in the absence of transcription. In the coupled system, cycloheximide concentrations completely arresting translation failed to inhibit RNA synthesis (data not shown).

Addition of VLPs to extracts of strain M816 (lacking endogenous M or L-A dsRNA) and incubation under coupled system conditions resulted in at least a fivefold stimulation of incorporation of [α-32P]UTP into TCA-precipitable material (data not shown), indicating the relatively low levels of endogenous RNA polymerase activity in micrococcal nuclease-treated translation extracts. Presumably at least some of the endogenous RNA polymerase activity is due to the presence of transcriptionally active L-BC dsRNA-containing VLPs in extracts of this strain. Electrophoretic analysis of the RNA products of the coupled system reaction run in the presence of added VLPs revealed that [α-32P]UTP was incorporated into the expected l and m transcripts as well as a more rapidly migrating band (500 to 800 nucleotides) of unknown origin, with lower levels of incorporation into L and M dsRNA (Fig. 3a, lane 1). No incorporation into the band of unknown origin was seen in the translation extract in the absence of added VLPs (Fig.

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Fig. 3. RNA products of the coupled system. (a) Coupled system reactions were run using a cell-free extract from strain M816 in the presence of [α-32P]UTP with or without added virions. Reactions were incubated for 7 h and were then extracted with phenol, chloroform/isoamyl alcohol, and precipitated with ethanol. Pellets were redissolved and subjected to electrophoresis in 1.2% agarose gels in the presence of ethidium bromide. Gels were dried and analysed by autoradiography. Lane 1 shows coupled system RNA products from a reaction containing added virions. Lane 2 is a parallel reaction without added virions. The origin (O), L dsRNA (L), M dsRNA (M), I transcript (I), m transcript (m) and a band of unknown origin (arrow) are indicated. (b) The reactions were run as in (a) and were then loaded onto a 10 to 40% linear sucrose gradient and subjected to ultracentrifugation at 80000 g in a SW41 rotor (Beckman) for 12 h at 4 °C. Thirty-drop fractions were collected from the bottom, followed by extraction with phenol and chloroform/isoamyl alcohol (24:1), and ethanol precipitation in the presence of carrier tRNA (10 μg). Samples were then subjected to electrophoresis as in (a). Numbers indicate gradient fractions where fraction 20 represents the top of the gradient; other symbols are as in (a). The basis for the apparent slower electrophoretic migration of transcript bands from the more rapidly sedimenting fractions is not known. No radioactive bands were seen in extracts of sucrose gradient fractions 1 to 10 subjected to similar analysis. A mixture of VLPs with a cell-free extract without further incubation was analysed on a parallel gradient, and showed a peak of virion-associated RNA polymerase activity (Welsh & Leibowitz, 1980) sedimenting in fractions 12 to 15. (c) Strand separation analysis of 32P-labelled RNA produced in the coupled system was performed on a 5% polyacrylamide gel (Thiele et al., 1984). Lane 1 contains gel-purified I transcript from fractions 17 and 18 of the sucrose gradient in (b); lane 2, gel-purified I transcript from fractions 13 to 15; lane 3, gel-purified L dsRNA from fractions 13 to 15; lane 4, L dsRNA from a separate coupled reaction purified by agarose gel electrophoresis. O indicates the origin, L⁻⁻ the negative strand and L⁺⁺ the positive strand of L-A dsRNA and Ln the poorly resolved separated strands of L-BC dsRNA.

RNA was extracted from a non-radioactive coupled system reaction, fractionated by denaturing agarose gel electrophoresis and transferred to nylon membranes for Northern blot analysis (Maniatis et al., 1982). Upon hybridization with radioactive dsRNA probes, it was observed that the 4.9 kb band corresponding to both L-A dsRNA and I transcript hybridized to 32P-labelled L-A dsRNA, and the 1.8 kb band corresponding to both M dsRNA and m transcript hybridized to the 32P-labelled M dsRNA probe. Similar specific hybridization was observed using 5′-32P-labelled oligonucleotide probes complementary to positions 241 to 265 (probe sequence 5′ TACACCTGCATCGCTCGCTACGAAT 3′) from the 5′ terminus of the positive strand of M dsRNA, or

3a, lane 2) or in standard VLP transcription reactions (data not shown).

As shown in Fig. 3(b), most of the radioactive L-A and M dsRNA produced in a coupled system reaction remained associated with VLPs throughout sucrose gradient sedimentation (fractions 12 to 15), whereas the I and m transcript products were associated with the VLP peak and with free RNA at the top of the gradient (fractions 17 to 20). The uncharacterized low M₁ RNA band (arrow) was found exclusively at the top of the sucrose gradient. The new band was not retained on an oligo(dT)-cellulose column, unlike the m transcript (Hannig et al., 1984) or the subgenomic m₅ transcript produced in vivo (Bostian et al., 1983).
positions 372 to 396 (probe sequence 5' GTGCCACGT-GATTGTCATACCAACG 3') from the 5' terminus of the positive strand of L-A dsRNA. However, none of these four probes hybridized to the low M, RNA produced in the coupled system reaction (data not shown). Since incorporation into this RNA requires both VLPs and the micrococcal nuclease-treated extract, and the RNA fails to hybridize to oligo(dT), M or L-A dsRNA or related probes, this product may represent the transcript of some other virus or plasmid which requires both the VLP preparation and extract for RNA polymerase activity.

The RNA products of the coupled system, purified by sucrose gradient centrifugation followed by agarose gel electrophoresis of RNA from gradient fractions (Fig. 3b), were further analysed by strand separation PAGE. The agarose gel-purified 1 transcript from gradient fractions 13 to 15 or 17 to 18 (Fig. 3b) comigrated with the positive strand of L-A dsRNA (Fig. 3c, lanes 2 and 1, respectively). Upon strand separation, the L dsRNA purified by agarose gel electrophoresis of fractions 13 to 15 of the sucrose gradient (Fig. 3b) or L dsRNA purified by agarose gel electrophoresis of a separate coupled system reaction (Fig. 3a), showed radioactivity predominantly in the negative-polarity strand of L-A dsRNA (Fig. 3c, lanes 3 and 4), and radioactivity in the positive-polarity strand was clearly detectable in the L dsRNA gel-purified from the coupled system reaction (Fig. 3c, lane 4). The small amount of L dsRNA recovered from the VLPs on the sucrose gradient (Fig. 3b) was insufficient to determine whether this positive-stranded material was present in VLPs (Fig. 3c, lane 3). This result indicates that both major capsid protein and positive-stranded transcripts of L-A dsRNA are being produced in the coupled system, with lower levels of dsRNA synthesis including production of the expected minus strands of L-A dsRNA and some positive-strand synthesis.

Previously, radioactive nucleotides incorporated into L-A dsRNA in transcription reactions were shown to be entirely in the negative strand (Williams & Leibowitz, 1987), which is consistent with the incorporation being due to low levels of VLPs in the preparation containing only positive strands of L-A with associated replicase activity catalysing negative-strand synthesis (Esteban & Wickner, 1986; Fujimura et al., 1986; Fujimura & Wickner, 1988b). In the coupled system, low levels of incorporation are also observed in positive strands of L-A dsRNA (Fig. 3c), a result not previously reported. This incorporation could potentially be due to the use of newly synthesized positive strands as templates for negative-strand synthesis. Free positive strands were previously shown to be templates for negative-strand synthesis by virions apparently lacking endogenous dsRNA (Fujimura & Wickner, 1988b). Recovery of VLP-associated dsRNA from sucrose gradient analysis of coupled reactions was insufficient to determine whether the radioactive positive strands of dsRNA were present. However, this result raises the possibility that modification of the coupled system might provide a cell-free assay for dsRNA replication or RNA packaging. Such an assay would be useful in studying viral and host cell mutants defective in these processes (reviewed by Wickner, 1986).

As discussed above, efficient expression of viral proteins in the coupled system may mimic in vivo coupling of transcription and translation of cytoplasmic killer virus. It is not known how the expression of apparently uncapped (Hannig et al., 1984; Nemeroff & Bruenn, 1987; Welsh & Leibowitz, 1980) transcripts is achieved in this eukaryotic system, and whether initiation of translation of nascent viral transcripts proceeds by a mechanism distinct from that used for the capped transcripts of nuclear genes of yeast.

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