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SUPPLEMENTAL DATA

The influence of eEF2 on dissociation of post-termination ribosomes into subunits.

It has recently been reported that in the presence of ATP, yeast eEF2 promotes a transient dissociation of empty yeast 80S ribosomes into subunits (Demeshkina et al., 2007). In light of these recently reported data, we investigated the influence of eEF2 on dissociation of mammalian post-TCs. Since GTP inhibited the dissociating activity of yeast eEF2 (Demeshkina et al., 2007), post-termination complexes were obtained by incubating pre-TCs with puromycin rather than with release factors because the activity of eRF3 requires GTP hydrolysis. The use of puromycin would also obviate a second potential problem, which is that if eRF1/eRF3 remain bound to post-TCs, and if their binding site overlaps with that of eEF2, they would potentially mask any dissociating activity of eEF2. In the absence of initiation factors, eEF2 at a concentration of 370 nM did not split post-TCs into subunits irrespective of the presence of ATP or GTP (Figure S1A), which theoretically could be due to the reportedly transient nature of eEF2-mediated dissociation. However, eEF2 also did not influence the dissociation of post-TCs by eIF3 alone (Figure S1B) or with different combinations of eIFs 1, 1A and 3j (data not shown). As expected, eEF2 also did not influence dissociation of post-TCs assembled using release factors (data not shown). Although we therefore did not observe any effect of eEF2 on dissociation of post-TCs, kinetic analysis was outside the scope of this study, so an influence of eEF2 on dissociation of post-termination 80S ribosomes cannot be entirely excluded.

The influence of eIF3 concentration on dissociation of post-termination ribosomes into subunits.

Throughout the current study, the concentration of pre-TCs in reaction mixtures was maintained at ~2nM, a level that was determined by the procedure used for preparation of pre-TCs. Thus, after assembly, pre-TCs were purified on sucrose density gradients that had been loaded to maximum capacity, and were then used in termination/recycling reactions directly following the dilution of peak fractions with the buffer in order to reduce the sucrose concentration. The concentration of pre-TCs in reaction mixtures could be increased either by concentration using centricons or by

pelletting by centrifugation of initial gradient fractions. However, both procedures could potentially compromise the integrity of pre-TCs (either by significantly lengthening the duration of purification in the case of concentration by centricons, or due to shear stresses during an additional centrifugation step) and therefore were not included in the pre-TC preparation procedure. The binding constants for factors and pre-TCs are not known. However, the facts that eIF3j enhances 40S/eIF3 association (Fraser et al., 2004) and that the dissociation constant for 40S/eIF3j interaction is 6 nM (Fraser et al., 2007) indicate that the dissociation constant for the 40S/eIF3 interaction is most likely substantially higher than 6 nM. Taking this conclusion and the relatively low concentration of pre-TCs in reaction mixtures into account, the concentrations of initiation factors were elevated 5-10 fold compared to their concentrations in rabbit reticulocyte lysates (which can be deduced by their abunadance relative to ribosomes; Duncan and Hershey, 1983; Meyer et al., 1982; Mengod and Trachsel, 1985) to ensure that reactions occurred with maximum efficiency and at maximum rate. As a result, the ratio between eIF3 and pre-TCs was ~100:1. Although estimates of the ratio between eIF3 and ribosomes in RRL and HeLa cells vary from 0.3 – 0.5 up to 1-2 (Duncan and Hershey, 1983; Meyer et al., 1982; Mengod and Trachsel, 1985) the cellular concentration of pre-TCs is not known and we therefore cannot comment on the relationship between the ratio used in our experiments and that in living cells. Although at the chosen concentrations of pre-TCs and initiation factors, a 10-15 minute incubation period was sufficient for termination, recycling and subsequent initiation to occur in a system with a complete set of initiation factors, the facts that eIF3 alone promoted dissociation of only ~45% of post-termination ribosomes into subunits (Figure 3B of the main text) and that its dissociating activity was strongly enhanced by eIF3j (Figure 3C of the main text), which stimulates 40S/eIF3 association (Fraser et al., 2004), suggest that the concentration (250 nM) of eIF3 that was used for experiments shown in Figure 3 might not be saturating for dissociation of post-TCs by eIF3 alone and that elevation of eIF3's concentration might increase the efficiency of dissociation. Consistently, a three-fold increase in the concentration of eIF3 from 250 to 750 nM led to an increase in

dissociation of post-termination complexes of up to 60-65%, whereas a decrease in eIF3's concentration to 50 nM resulted in a strong reduction in dissociation of post-TCs (Figure S1C). The ability to enhance dissociation of post-TCs by eIF3 alone by increasing the concentration of the factor indicates that stimulation by eIF3j of the dissociating activity of eIF3 could at least in part be due to eIF3j's ability to stimulate 40S/eIF3 association (Fraser et al., 2004).

Recycling of post-termination complexes assembled on mRNA with a 12 amino acid open reading frame

Eukaryotic ribosomes reinitiate efficiently only after translating short ORFs. This implies that post-termination events after translation of long and short ORF may differ slightly. It has been suggested that some eIFs (e. g. eIF3) might remain associated with ribosomes after translation of short ORFs (Pöyry et al., 2004). Although all pre-TCs described here were purified by sucrose gradient centrifugation, which removes all weakly associated factors, and recycling of pre-TCs assembled on MVHL-STOP mRNA required addition of eIF3 and other eIFs, we nevertheless investigated recycling of pre-TCs assembled on $M(VF)_5L$-STOP mRNA (Figure S2A), in which the ORF was extended to 12 codons, by following dissociation of mRNA. Efficient, uninterrupted elongation on long ORFs in the *in vitro* reconstituted system is complicated by the unavailability of individual native mammalian tRNAs and because total tRNA at concentrations required for translation of long ORFs is inhibitory in this system. However, systematic testing of various mRNAs revealed that initiation complexes assembled on $M(VF)_5L$-STOP mRNA could be almost quantitatively transferred into pre-TCs upon addition of eEFs and total aminoacylated tRNA (Figure S2B).

Incubation of pre-TCs assembled on $[^{32}P]M(VF)_5L$-STOP mRNA with eRF1•eRF3•GTP released ~35% of mRNA just as with MVHL-STOP mRNA (Figure S2C, open diamonds; Figure S2D, lane 2). After incubation with eRF1/eRF3 and eIF3, ~30% of mRNA was bound to post-termination 80S ribosomes, ~25% was bound to 40S subunits and the rest was engaged in mRNPs (Figure S2C, triangles; Figure S2D, lanes 3, 5). Incubation with eRF1/eRF3 and eIFs 3, 1, 1A and 3j

resulted in very little association of mRNA with 80S ribosomes or recycled 40S subunits (Figure S2C, circles; Figure S2D, lanes 4, 6). Again, as for MVHL-STOP mRNA, only 40S subunit-containing complexes obtained in the absence of eIF1 yielded a toe-print stop +16nt from the P site Leu codon (Figure S2D, lane 5). In conclusion, eIFs 3, 1, 1A and 3j acted identically on post-TCs assembled on mRNAs with 4 or 12 amino acid-long ORFs.

Supplemental Experimental Procedures

Plasmids

To construct a vector for $M(VF)_5L$-STOP mRNA, DNA sequence flanked by an upstream T7 promoter was inserted between *Pst*I and *Sma*I restriction sites in pUC57 (Celtek Genes). mRNA was transcribed using T7 polymerase from a *Sma*I-digested plasmid. For mRNA dissociation experiments, $[^{32}P]M(VF)_5L$-STOP mRNA ($1.5x10^6$ cpm/μg) was transcribed in the presence of $[\alpha^{32}P]$ATP (222 Tbq/mmol).

References

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Supplemental Figures

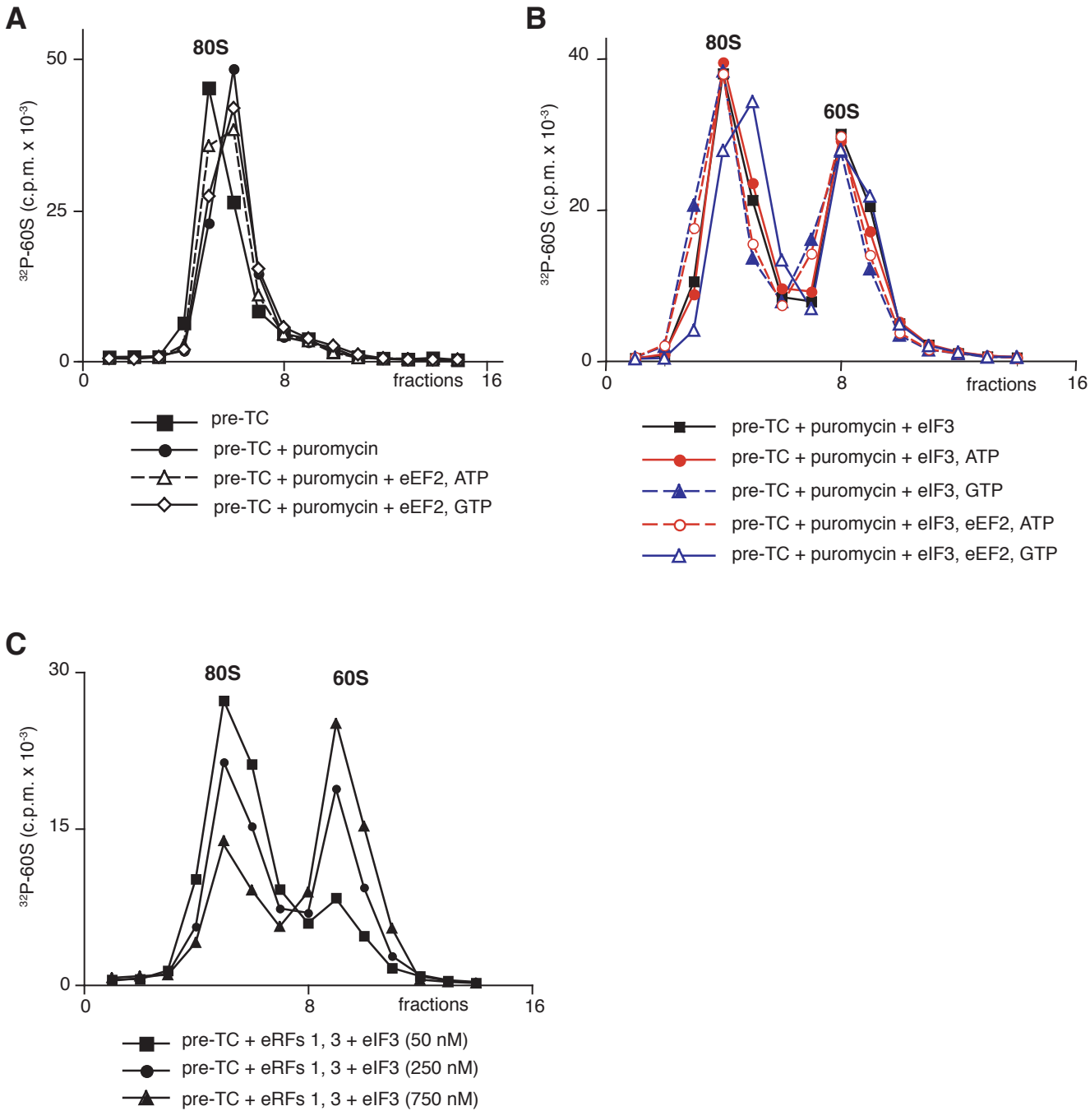
Figure S1. Dissociation of post-TCs into subunits at different concentrations of eIF3 or in the presence of eEF2.

Dissociation of pre-TCs, assembled on MVHL-STOP mRNA with [^{32}P]60S subunits, after their incubation with eRFs and (A, B) with eIF3, eEF2, ATP and GTP, as indicated, or (C) with different concentrations of eIF3, assayed after sucrose gradient centrifugation by Cerenkov counting. The positions of 60S subunits and 80S ribosomes are indicated.

Figure S2. Dissociation of mRNA from post-TCs assembled on mRNA with a 12 amino acids-long ORF.

(A) The structure of M(VF)$_5$L-STOP mRNA. (B) Toe-printing analysis of 48S complexes and pre-TCs assembled on M(VF)$_5$L-STOP mRNA. Components of reaction mixtures are indicated. The position of ribosomal complexes are shown relative to the mRNA codon in the P site. Lanes C, T, A, G depict cDNA sequences corresponding to M(VF)$_5$L-STOP mRNA. (C) Ribosomal association of

[^{32}P]M(VF)$_5$L-STOP mRNA after incubation of pre-TCs with different combinations of release and initiation factors, assayed by sucrose density gradient centrifugation. The positions of 40S subunits and 80S ribosomes are indicated. (D) Toe-printing analysis of 40S and 80S ribosomal peaks (shown on panel C). The positions of full-length cDNA and of toe-prints that correspond to different ribosomal complexes are indicated. Lanes C, T, A, G depict cDNA sequences corresponding to M(VF)$_5$L -STOP mRNA.



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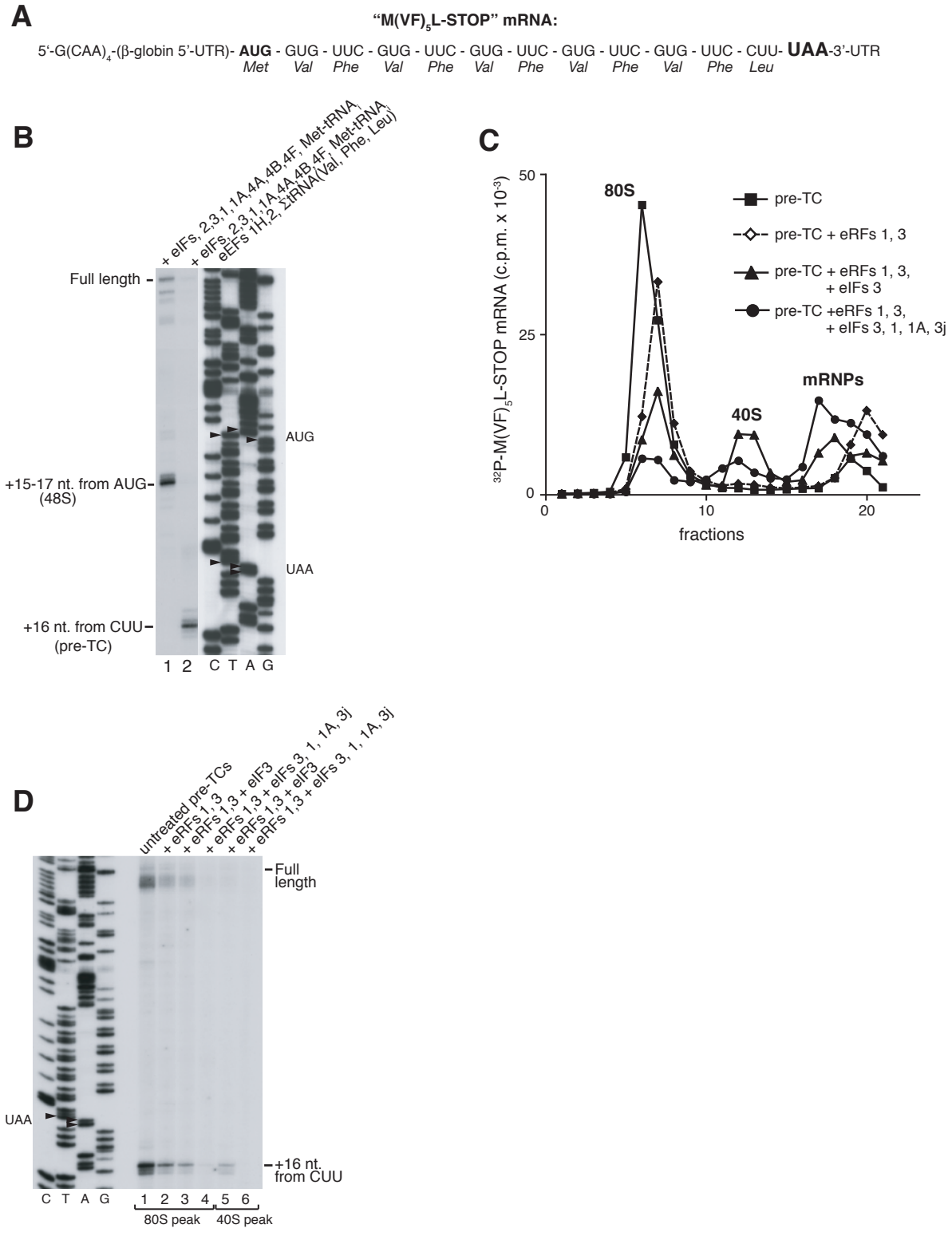
(Supplemental data)



Pisarev et al., Figure S2
(Supplemental data)