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The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability

S.Jimeno, A.G.Rondón, R.Luna and A.Aguilera[1]

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41012 Sevilla, Spain

[1]Corresponding author
e-mail: aguilo@us.es

The THO complex is a multimeric factor containing four polypeptides, Tho2, Hpr1, Mft1 and Thp2. Mutations in any of the genes encoding THO confer impairment of transcription and a transcription-dependent hyper-recombination phenotype, suggesting that THO has a functional role in gene expression. Using an *in vivo* assay developed to study expression of long and G+C-rich DNA sequences, we have isolated *SUB2*, a gene involved in mRNA splicing and export, as a multicopy suppressor of the gene expression defect of *hpr1*Δ. Further investigation of a putative functional relationship between mRNA metabolism and THO revealed that mRNA export mutants *sub2*, *yra1*, *mex67* and *mtr2* have similar defective transcription and hyper-recombination phenotypes as THO mutants. In addition, THO becomes essential in cells with a defective Mex67 mRNA exporter. Finally, we have shown that THO has the ability to associate with RNA and DNA *in vitro*. These results indicate a functional link between the processes of elongation and metabolism of nascent mRNA mediated by THO and mRNA export proteins, which have important consequences for the maintenance of genome stability.

Keywords: Hpr1–Tho2/Mex67–Mtr2/RNA metabolism/Sub2-Yra1/THO complex

Introduction

Eukaryotic transcriptional elongation is a complex nuclear process in which RNA polymerase II (RNAPII) has to overcome situations derived from transient pausing caused by regulatory signals. This is achieved with the help of negative and positive transcriptional elongation factors (for a review, see Lee and Young, 2000; Zorio and Bentley, 2001). As transcription proceeds, the nascent RNA molecule has to undergo proper maturation, including 5′-end methylguanine capping, splicing, 3′-end cleavage and poly(A)$^+$ addition (see Hirose and Manley, 2000; Proudfoot, 2000). Capping enzymes are directed to the 5′ ends of nascent RNAs by binding to the phosphorylated RNAPII C-terminal domain (CTD; Cho *et al*., 1997; McCracken *et al*., 1997a) and act when the nascent pre-mRNA is ~25 nucleotides (nt) long (Rasmussen and Lis, 1993). Splicing also occurs co-transcriptionally (Beyer and Osheim, 1988). Indeed, it has been shown that splicing factors associate with the transcription elongation complex via the CTD of RNAPII (Steinmetz, 1997). Poly(A)$^+$ addition is also coupled with transcription elongation as supported by the observations that the cleavage polyadenylation stimulatory factor (CPSF), responsible for recognizing the poly(A)$^+$ signal sequence, is associated with the RNAPII CTD during elongation (Dantonel *et al*., 1997) or that cleavage of the poly(A)$^+$ site is dependent on the CTD of RNAPII (McCracken *et al*., 1997b).

In vivo cross-talks between transcription and splicing, poly(A)$^+$ addition and RNA export have also been reported (reviewed in Hirose and Manley, 2000; Cramer *et al*., 2001; Zenklusen and Stutz, 2001). These include suggestions that a truncation of the RNAPII CTD leads to the accumulation of significant amounts of unspliced pre-mRNAs in mammalian cells (Misteli and Spector, 1999); that human β-globin pre-mRNAs defective in either splicing or 3′-end formation are retained at the site of transcription (Custodio *et al*., 1999); that the Aly/REF RNA export factor accumulates in splicing factor-containing nuclear speckles (Zhou *et al*., 2000); or that different blocks in yeast mRNA nuclear export leads to accumulation of hyper-adenylated transcripts at the site of transcription (Jensen *et al*., 2001). All these results are consistent with the present view that transcription of a DNA segment involves not only the elongating RNAPII complex and elongation factors, but also RNA-associated protein complexes involved in RNA processing and export (Dreyfuss *et al*., 1993).

Export of mRNA via the nuclear pore is a conserved process among eukaryotes that is catalysed by a number of factors (see Cullen, 2000). These include the Aly/REF factor, the yeast orthologue of which is Yra1 (Portman *et al*., 1997; Strässer and Hurt, 2000; Stutz *et al*., 2000). Yra1 is an RNA annealing protein (Portman *et al*., 1997) that associates with chromatin in a transcription-dependent manner (Lei *et al*., 2001). It is found in association with the putative RNA helicase UAP56/Sub2, which is also involved in both splicing (Kistler and Guthrie, 2001; Libri *et al*., 2001) and export (Luo *et al*., 2001; Strässer and Hurt, 2001). Other essential export factors are the TAP/Mex67 and p15/Mtr2 proteins, which form a heterodimeric RNA exporter that interacts with Yra1/ALY and drives the mRNP through the nuclear pore (Santos-Rosa *et al*., 1998; Katahira *et al*., 1999; Strässer and Hurt, 2000). It binds *in vivo* to poly(A)$^+$ RNA, contacts the nuclear pore and shuttles between the nucleus and the cytoplasm (Segref *et al*., 1997).

THO was identified as a four-protein complex containing proteins encoded by *THO2* and *HPR1*, two genes previously identified by hyper-recombination mutations (Aguilera and Klein, 1990; Piruat and Aguilera, 1998), and *MFT1* and *THP2* (Chávez *et al*., 2000). The null mutations of the four genes are viable and yield similar phenotypes of

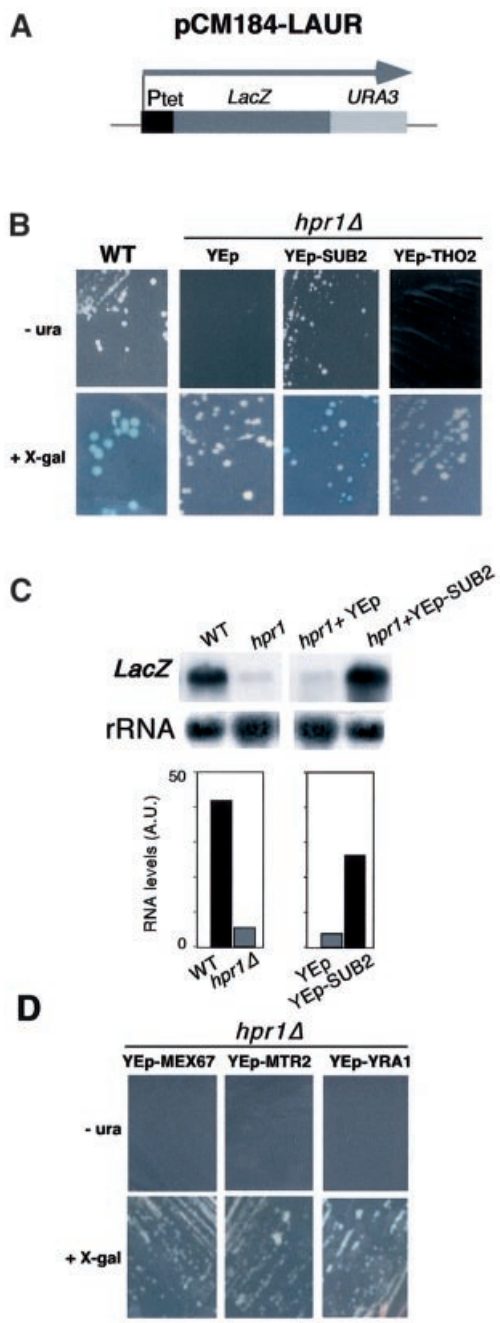

Fig. 1. Suppression of the transcriptional effect of *hpr1* by overexpression of *SUB2*. (**A**) Scheme of the *lacZ–URA3* fusion fragment under the control of the *tet* promoter used. (**B**) Phenotypic analysis of wild-type (W303-1A) and *hpr1*Δ (U678-1C) strains, transformed with either vector YEp351 or plasmids YEp351-SUB2 and YEp351-THO2 containing *SUB2* and *THO2*, respectively. The capacity of each strain to form colonies on synthetic medium lacking uracil after 4 days at 30°C is shown on the top. On the bottom is the capacity of each strain to express β-galactosidase from plasmid pCM184-LAUR on complete synthetic medium, as determined by a colour assay. (**C**) Northern analysis of the expression of the *Ptet::lacZ–URA3* fusion construct. RNA was isolated from mid-log phase cells carrying pCM184-LAUR grown in synthetic medium. As ^{32}P-labelled DNA probe we used a 3 kb *Bam*HI *lacZ* fragment and the internal 589 bp 28S rRNA fragment obtained by PCR. RNA levels in arbitrary units (A.U.) were obtained in a Fuji FLA 3000 and were normalized with respect to rRNA levels of each sample. (**D**) Phenotypic analysis of *hpr1*Δ (U768-1C) cells transformed with Yep351-YRA1, YEp13-MEX67 and YEp13-MTR2.

impairment of transcription elongation and transcription-dependent hyper-recombination between direct repeats (Prado and Aguilera, 1997; Piruat and Aguilera, 1998; Chávez *et al*., 2000). We have previously proposed that the THO complex has a functional role related to RNAPII transcription elongation. Whether this role is direct or indirect is not yet known. However, we cannot exclude the possibility that THO might also have a role in RNA metabolism beyond transcription. Indeed, it has been shown that *hpr1* cells show RNA export defects at 37°C (Schneiter *et al*., 1999). In addition, it has recently been shown that multiple copies of the putative RNA helicase Sub2 partially suppresses the hyper-recombination phenotype of *hpr1* cells and that *sub2* mutants are hyper-recombinant (Fan *et al*., 2001).

In this study we show that the THO complex associates with RNA and DNA *in vitro* and provide *in vivo* evidence that THO and RNA export factors are functionally related. We show that multicopy Sub2 suppresses the transcriptional defect of *hpr1* cells and that *sub2*, *yra1*, *mex67* and *mtr2* mutants show similar defective transcription and hyper-recombination phenotypes to THO mutants. These results suggest that there is a functional connection between transcription and RNA export mediated by THO and RNA export factors that plays an important role in genetic instability.

Results

Multicopy SUB2 suppresses the gene expression defect of hpr1Δ

In order to obtain more insights into the functional role of the THO complex on gene expression, we searched for genes which could potentially have an overlapping function with THO. We first developed a new genetic assay for the analysis of gene expression. We constructed the centromeric plasmid pCM184-LAUR containing a 4.15 kb *lacZ–URA3* translational fusion under the control of the P_{tet} promoter, which is active under all conditions used in this study (Figure 1A). We expected that *hpr1* mutants would not express this construct, because *hpr1*Δ mutants cannot express long and G+C-rich DNA sequences such as *lacZ* fused to a strong promoter (Chávez *et al*., 2001). As predicted, *hpr1* strains transformed with pCM184-LAUR were unable to form colonies on SC-ura and lacked β-galactosidase activity (Figure 1B).

From ~60 000 transformants of the multicopy library MW90 (Waldherr *et al*., 1993) plated on SC-ura, we selected 30 that recovered the capacity to grow in the absence of uracil. Isolation and restriction analysis of the plasmids carried by these transformants defined eight different types of plasmids. Five carried overlapping genomic DNA fragments containing the whole *SUB2* gene, whereas the other three carried overlapping inserts containing the whole *THO1* gene, as determined by DNA sequencing. Sub2 is a putative RNA helicase involved in mRNA splicing and export (Jensen *et al*., 2001; Kistler and Guthrie, 2001; Libri *et al*., 2001), whereas Tho1 has N-terminal homology with the human RNA-binding protein hnRNPU (Fackelmayer and Richter, 1994). As we have previously described Tho1 as a suppressor of the

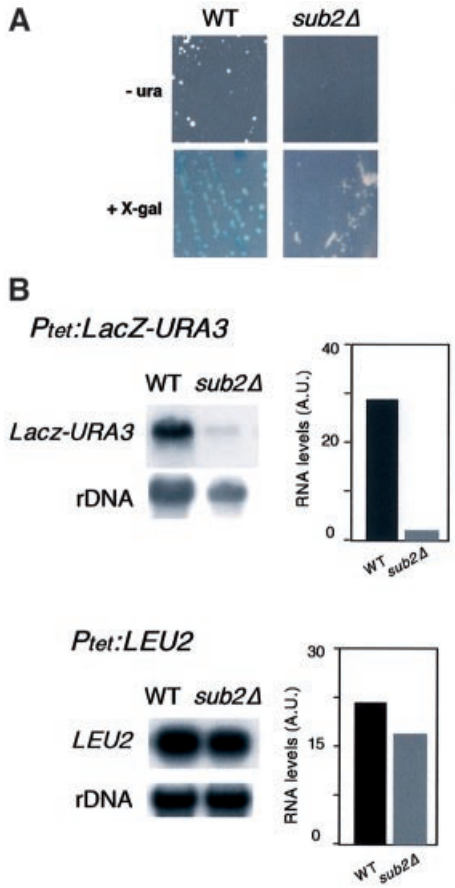

Fig. 2. Genetic and molecular analysis of transcription in *sub2*Δ strains. (**A**) Phenotypic analysis of wild-type (CEN.PK) and *sub2*Δ isogenic strains. The capacity of each strain to form colonies in synthetic medium lacking uracil after 4 days at 30°C is shown on the top. On the bottom is the capacity of each strain to express β-galactosidase from plasmid pCM184-LAUR on complete synthetic medium, as determined by a colour assay. (**B**) Northern analysis of the expression of the *Ptet::lacZ–URA3* and *Ptet::LEU2* fusion constructs in yeast transformants carrying plasmids pCM184-LAUR and pCM189-LEU2, respectively. As ^{32}P-labelled *LEU2* probe we used the *Cla*I–*Eco*RV *LEU2* internal fragment. Other details as in Figure 1.

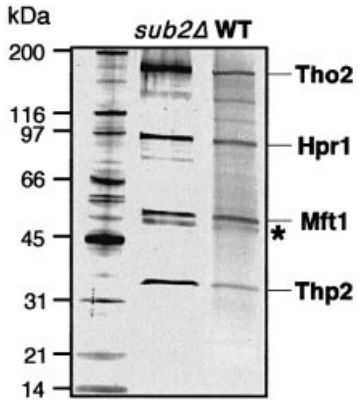

Fig. 3. Silver-stained SDS–PAGE of Tho2-TAP-tagged THO complex purified from wild-type and *sub2*Δ cells. The novel band (*) co-purifying with the TAP-tagged THO complex corresponds to Tex1 (Strässer *et al*., 2002).

transcriptional defect of *hpr1*Δ (Piruat and Aguilera, 1998), we did not pursue the work on this gene.

Figure 1B shows that *hpr1*Δ cells transformed with any of these multicopy plasmids carrying *SUB2* were able to grow on SC-ura and to express β-galactosidase activity. We showed by northern analyses that these transformants transcribed *lacZ–URA3* at levels close to wild-type cells (Figure 1C). We provide, therefore, evidence that Sub2 in multicopy can substitute for the role of Hpr1 in gene expression *in vivo.* As shown recently (Fan *et al*. 2001), we have confirmed that multicopy *SUB2* partially suppresses *hpr1* hyper-recombination (data not shown).

It is worth noting that in this search we did not isolate *THO2*, which we had previously identified as a multicopy suppressor of the temperature-sensitive (ts) and gene expression-defect phenotype of *hpr1* cells (Piruat and Aguilera, 1998). Indeed, we show that multicopy *THO2* cannot re-establish expression of the *lacZ–URA3* in *hpr1* cells (Figure 1B). This indicates that multicopy *SUB2*, but not *THO2*, can either fully substitute or bypass the need for Hpr1 and presumably the THO complex in the cell. Failure to isolate Hpr1 suggests that our genetic search for suppressors was not saturated.

sub2 mutants show reduced transcription efficiency of lacZ sequences

As the Sub2 function seemed to be linked to that of the THO complex, we decided to investigate whether *sub2* showed gene expression phenotypes similar to THO mutants, i.e. reduced capacity to transcribe long and G+C-rich DNA sequences such as *lacZ.*

We first determined the effect of *sub2*Δ on transcription of the P_{tet}*::lacZ–URA3* fusion construct. For this, we used a CEN-PK6 genetic background in which the null *sub2*Δ mutation was viable (EUROSCARF, Frankfurt, Germany). Figure 2A shows that *sub2*Δ strains containing the P_{tet}*::lacZ–URA3* fusion were unable to either form colonies on SC-ura or to express β-galactosidase activity. As can be seen in Figure 2B, the transcripts were accumulated in the mutants at 7% of the wild-type levels. However, *sub2*Δ mutants were able to transcribe a P_{tet}*::LEU2* construct at wild-type levels. This indicates that *sub2* cells are impaired in their ability to transcribe *lacZ* but not in their ability to promote transcription from P_{tet} or to transcribe the *LEU2* sequence, a short, low G+C-content sequence. This resembles the effect of *hpr1*, *tho2*, *mft1* and *thp2* mutants on transcription (Chávez and Aguilera, 1997; Piruat and Aguilera, 1998; Chávez *et al*., 2000).

A complete THO complex is assembled in sub2Δ strains

The similarity of transcription and recombination phenotypes between *sub2*Δ and the mutants of the *THO* complex prompted us to ask whether the THO complex was unstable or was not formed in *sub2*Δ mutants. In order to purify the THO complex from wild-type and isogenic *sub2*Δ strains, we replaced the wild-type copy of *THO2* with a TAP-tagged wild-type copy. As can be seen in Figure 3, a full THO complex containing Tho2, Hpr1, Mft1 and Thp2 could be purified in both wild-type and *sub2*Δ strains. Therefore, we can conclude that the transcription and recombination phenotypes of *sub2*Δ is

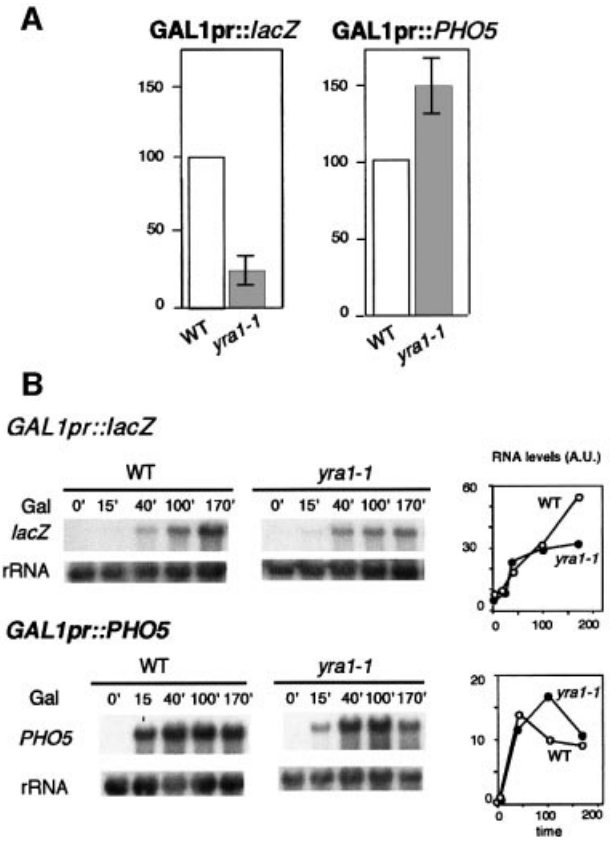

Fig. 4. Expression analysis of *GAL1pr::lacZ* and *GAL1pr::PHO5* constructs in wild-type (RS453a) and *yra1-1* strains. (**A**) β-galactosidase and acid phosphatase activities of strains transformed with plasmids p416-GAL1–lacZ and pSCh202. The percentage value of activity is shown with respect to wild type (100%). Each value represents the average of two to three different transformants. Only data of induced expression are given (2% galactose). Under repressed conditions (2% glucose) values were below detection levels in all cases. (**B**) Northern analyses of *lacZ* and *PHO5* mRNAs driven from the *GAL1* promoter. Mid-log phase cells were diluted in 3% glycerol–2%lactate SC-ura fresh media to an OD_{600} of 0.5 and incubated for 16 h. Galactose was then added and samples were taken at different times, as specified. As DNA probes we used the 0.9 kb *Eco*RV internal fragment of *PHO5*. For the *lacZ* and *rRNA* probes and other details see Figure 1.

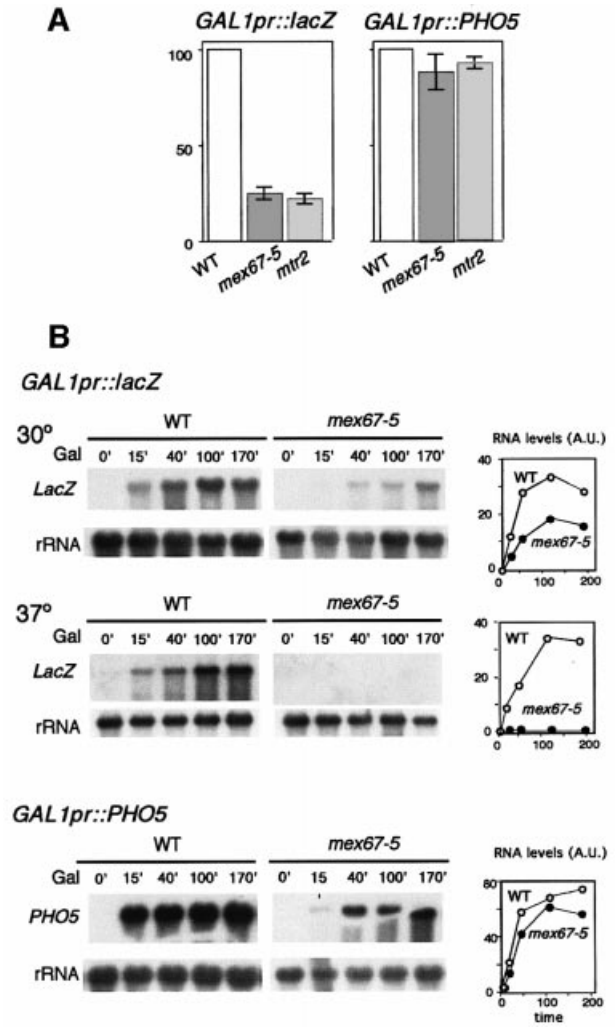

Fig. 5. Expression analysis of *GAL1pr::lacZ* and *GAL1pr::PHO5* constructs in *mex67-5* (WMC1-1A), *mtr2-ts26* and their isogenic wild-type strains (W303-1A and RS5453a, respectively). The experiments were carried out either at 30°C or after shifting cells to 37°C 30 min before activating transcription in media containing galactose. Details as in Figure 4.

not caused by a side-effect of the absence of Sub2 on the formation or stability of the THO complex.

yra1, mtr2 and mex67 conditional mutants are also impaired in lacZ transcription

Since *hpr1*Δ and *sub2* mutants are also defective in RNA export (Schneiter *et al*., 1999; Strässer and Hurt, 2001), we next investigated whether the transcriptional and recombination phenotypes observed in THO and Sub2 mutants were also observed in previously identified mRNA export mutants. We analysed the ts mutations *yra1-1*, *mex67-5* and *mtr2-26*. Yra1 is the yeast Aly/REF factor, an essential nuclear RNA annealing protein involved in RNA export that forms a complex with Sub2 (Portman *et al*., 1997; Strässer and Hurt, 2001). Mex67 and Mtr2 form one essential heterodimeric factor that functions downstream of Yra1 in mRNA export and are recruited to the mRNA via Yra1 (Strässer and Hurt, 2000).

As recombination could only be assayed under active growth conditions, we did the *in vivo* analyses at semi-permissive temperature (30°C). In addition, and in order to carry out a more precise study of the effect on transcription, we used the *GAL1pr::lacZ* and *GAL1pr::PHO5* assays (Chávez *et al*., 1997), which permitted us to determine the kinetics analysis of transcription through a long, G+C-rich open reading frame (ORF) (*lacZ*) and a short, G+C-low content ORF (*PHO5*).

Figure 4 shows that *yra1-1* reduced β-galactosidase activity to 25% of the wild-type levels, whereas expression of acid phosphatase in *yra1-1* mutants was slightly higher than in wild-type cells. Similar results were obtained by northern analysis. The kinetics of transcription activation of *GAL1pr::lacZ* showed reduced levels of activation in *yra1-1* with respect to wild type. The kinetics of transcription activation of *GAL1pr::PHO5* was even faster in *yra1-1* strains as compared with wild type, an effect that we have previously observed in other mutants of the THO complex such as *thp2*Δ (Chávez *et al*., 2001).

Figure 5 shows that *mex67-5* and *mtr2-26* ts mutations reduced the levels of β-galactosidase activities to 24 and 23% of the wild-type levels, respectively. However,

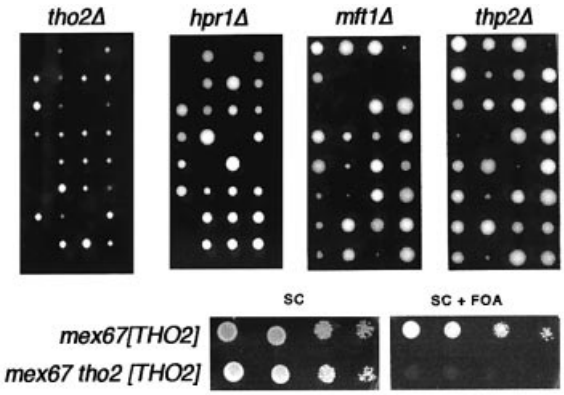

Fig. 6. Genetic analysis of synthetic lethality of *mex67-5* in combination with mutations of the THO complex. Tetrad analysis of heterozygous diploid strains obtained by crossing *mex67-5* strains with either *tho2*Δ, *hpr1*Δ, *mft1*Δ or *thp2*Δ null mutants is shown (top). In *hpr1*Δ and *tho2*Δ the double mutant combinations were unable to germinate. In contrast to the single mutant *mex67-5*, the double mutant *mex67-5 tho2*Δ strain WMT2 transformed with plasmid pRS316THO2 containing a wild-type copy of *THO2* was unable to form colonies on SC + 500 μg/ml of FOA (bottom).

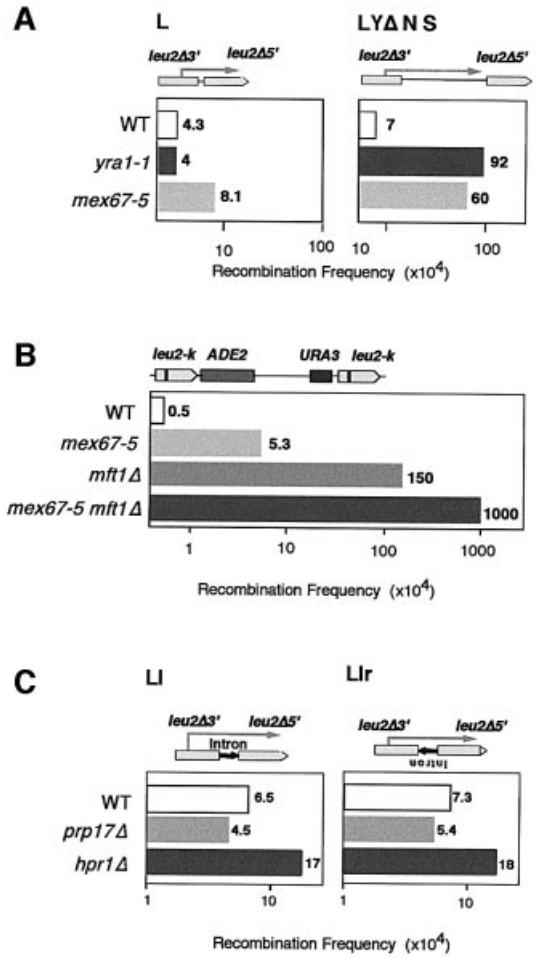

Fig. 7. Recombination analyses of mutants affected in RNA metabolism. (**A**) Recombination frequencies of wild-type (RS453a) and *yra1-1* and *mex67-5* mutant strains transformed with plasmids pRS316LΔNS and pRS316L. The scheme of each system is shown. Arrows indicate the transcripts driven from the *LEU2* promoter. For fluctuation tests, independent colonies were obtained from SC-ura and recombinants were selected in SC-leu-ura. (**B**) Recombination analysis of wild-type (MFM67-8A), *mex67-5* (WML-6D), *mft1*Δ (MFM67-13A) and *mex67 mft1*Δ (MFM67-12C) strains using the chromosomal direct-repeat assay *leu2-k::URA3-ADE2::leu2-k*. (**C**) Recombination analysis of wild-type (MHJ130), *hpr1*Δ (U768-1C) and *prp17*Δ (MHJ131L) using the LI and LIr direct-repeat recombination assays containing the *ACT1* intron between the 600 bp *leu2* repeats in both the processed and unprocessed orientations, respectively. For fluctuation tests, independent colonies were obtained from SC and recombinants were selected in SC+FOA or SC-leu. The median frequency of six to 12 independent cultures is given in each case. All experiments were performed at 30°C.

expression of acid phosphatase was similar in mutant and wild-type cells. These results were confirmed for the *mex67-5* mutation by northern analyses. The kinetics of transcription activation of *GAL1pr::lacZ* showed reduced levels of transcription in *mex67-5* with respect to wild type. In contrast, the kinetics of transcription of *GAL1pr::PHO5* was similar in both *mex67-5* and wild type. These experiments were repeated in conditions in which cells were shifted to 37°C to fully inactivate Mex67-5, 30 min prior to the analysis of the kinetics of activation of the *GAL1–lacZ* construct. Figure 5 shows that after the temperature shift *lacZ* mRNA was absent in *mex67-5* mutants, indicating that the effect of *mex67-5* on *lacZ* transcription was not secondary.

In contrast to SUB2, however, multicopy *YRA1*, *MEX67* and *MTR2* were not able to suppress the incapacity of *hpr1*Δ cells to express the *lacZ::URA3* construct as determined by the inability of *hpr1*Δ transformants to grow on SC-ura or to develop β-galactosidase activity (Figure 1D).

Our results therefore indicate that mutations in different mRNA export genes have a negative effect on transcription that is qualitatively similar to those of THO mutants.

mex67-5 is synthetic lethal with hpr1Δ and tho2Δ

The previous results indicating similar expression defect phenotypes of RNA export mutants to those of the THO complex, and that Sub2 can suppress in multicopy the gene expression defects of *hpr1* mutants, open the possibility for a genetic and functional interaction between mRNA export factors and the THO complex. To explore this possibility, we investigated whether double mutants carrying a mutation in an RNA export factor, such as *mex67-5*, and a mutation in either gene of the THO complex were synthetic lethal. Figure 6 shows that *mex67-5* is synthetic lethal with *hpr1*Δ and *tho2*Δ, as determined by tetrad analysis. This result was confirmed by showing that *tho2 mex67-5* mutants transformed with a *URA3*-based plasmid carrying *THO2* were unable to form colonies on SC+FOA (Figure 6). Double mutants *mex67-5 mft1*Δ and *mex67-5 thp2*Δ, however, were viable but grew very poorly. The synthetic lethality and poor growth phenotypes of the double mutants of the THO complex and *mex67-5* confirm, therefore, that there is a genetic and functional interaction between THO and RNA export factors. Consistent with this, similar results have been obtained in a parallel study with *sub2* and *yra1* mutants (Strässer *et al*., 2002).

RNA export mutations confer a hyper-recombination phenotype

We next analysed the effect of *yra1-1* and *mex67-5* on the frequency of recombination of two different direct-repeat

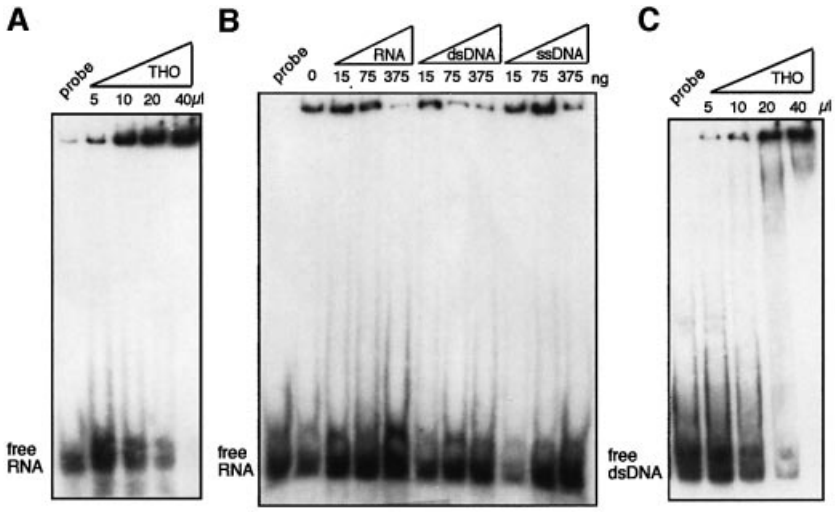

Fig. 8. *In vitro* RNA and DNA binding activities of the His-tagged Tho2 purified THO complex. (**A**) RNA binding assay using the 90 bp ^{32}P-labelled RNA obtained from the pBluescript SK polylinker and increasing amounts of a preparation of purified complex. (**B**) Competition assays of the RNA-binding activity of the THO complex using as competitor increasing amounts of the same 90mer RNA, a 60mer ssDNA and a 200 bp dsDNA. (**C**) DNA binding assay using a 200 bp 5′-end ^{32}P-labelled, PCR-amplified dsDNA and increasing amounts of the same purified THO complex used in (A).

constructs, both based on 600 bp internal *LEU2* fragment used as repeat. We have previously shown that the first system, L, carrying no DNA fragment between the repeats, is not hyper-recombinant in *hpr1* mutants. The second, LYΔNS, carrying a 2.7 kb pBR322–*URA3* fragment between the repeats, is hyper-recombinant as a consequence of a defective transcription of pBR322 caused by *hpr1* (Prado *et al*., 1997). Similarly to the *hpr1*Δ mutation, *yra1-1* and *mex67-5* did not confer hyper-recombination in the L repeat, whereas recombination was increased 13-fold in *yra1-1* and 8.6-fold in *mex67-5* cells in the LYΔNS system, in which transcription has to proceed through the pBR322–*URA3* region. This relatively weak increase in recombination correlates with the weak effect on transcription.

As can be seen in Figure 7B, the double mutant *mex67-5 mft1*Δ, which was viable, shows an increase in recombination (2000-fold above wild-type levels) of the chromosomal *leu2-k::URA3–ADE2::leu2-k* repeat construct that was synergistic with respect to the increase in recombination observed in each single mutant (10- and 300-fold). This result suggests that both THO complex and RNA export factors have different but cumulative effects on the process of RNA metabolism and it is consistent with either synthetic lethality or poor growth phenotype of *mex67-5* in combination with either *tho2*, *hpr1*, *mft1* or *thp2*.

Hyper-recombination is not linked to RNA splicing

As Sub2 was first identified as an RNA splicing factor (Kistler and Guthrie, 2001; Libri *et al*., 2001), we wondered whether hyper-recombination could also be linked to RNA splicing. For this purpose, we developed a *leu2* direct-repeat system containing between the 600 bp *leu2* repeat units an *ACT1* intron in both orientations, so that the intron could be processed in only one (LI system) and not the other (LIr). We first confirmed by northern analysis that the intron was processed in the LI system and not in the LIr system, although not with 100% efficiency (data not shown). Figure 7C shows that in both wild-type and *hpr1* strains the frequency of recombination was similar regardless of the intron orientation. Similar results were obtained with a *prp17* mutation deficient in the 3′-end processing of introns (Dagher and Fu, 2001), regardless of the orientation of the *ACT1* intron. Therefore, we can conclude that RNA splicing is not associated with hyper-recombination.

The THO complex associates with RNA and DNA in vitro

Finally, we investigated whether the THO complex has the ability to associate with either RNA or DNA *in vitro*. For this we purified His_6-Tho2 tagged THO complex as described previously (Chávez *et al*., 2000). RNA band-shifting assays using a 90 nt long RNA molecule shows that THO binds RNA (Figure 8A). Binding is directly proportional to the amounts of THO used and is very stable, as it resists salt concentrations >600 mM (data not shown). The RNA binding ability could be competed with cold RNA and double-stranded DNA (dsDNA) and, to a minor degree, with cold single-stranded DNA (ssDNA) (Figure 8B). Direct DNA-band shifting assays using a labelled 200 bp dsDNA clearly showed that the THO complex can associate with dsDNA *in vitro* (Figure 8C).

We do not know whether there is a particular protein from the THO complex able to bind RNA *in vitro*. In any case, we know that THO belong to a complex larger than 2 MDa as determined by gel filtration experiments (Chávez *et al*., 2000). This is consistent with the recent observation that THO is present in a large protein complex containing Sub2, Yra1 and other proteins (Ho *et al*., 2002; Strässer *et al*., 2002). Indeed, we have shown by western analysis of the THO complex using anti-Sub2 and anti-Yra1 antibodies that this highly purified His_6-tagged THO complex also contains Sub2 migrating at the same position as Mft1, but not Yra1 (Strässer *et al*., 2002). Although Yra1 has been detected in other TAP-tagged purified THO

(Ho *et al*., 2002; Strässer *et al*., 2002), our results suggest that Yra1 binding to THO is not very strong. Whether Sub2 is responsible for RNA association of THO is not known. In any case, our results indicate that THO has the ability to associate with RNA and DNA, whether or not in association with other proteins. We also know that the THO complex is not stabilized by RNA, as RNase treatment of our THO complex does not alter its stability (data not shown).

Discussion

We provide evidence for a functional link between transcription and mRNA metabolism, mediated by the THO complex and RNA export factors. This conclusion is based on a number of observations. First, we have isolated *SUB2* as multicopy suppressor of the gene expression defect of *hpr1*Δ. Secondly, different mutations of the essential RNA export genes *SUB2*, *YRA1*, *MEX67* and *MTR2* show similar defective transcription and transcription-dependent hyper-recombination phenotypes such as THO mutants. Thirdly, the THO complex is essential for growth in yeast cells with a defective mRNA export factor Mex67. Finally, the THO complex in combination with Sub2 can associate with RNA and DNA *in vitro*. Altogether, these results open the possibility that the gene expression defect caused by mutants of the THO complex is linked to a defect in RNA metabolism during transcription that, in addition, yields a recombinogenic structure at the DNA.

We show that *hpr1*Δ cells, mutated in the THO complex, are unable to express a *lacZ–URA3* transcript placed under control of the P_{tet} promoter. Using this assay we have isolated Tho1 and Sub2, as multicopy suppressors of the gene expression defect of *hpr1*Δ cells. Tho1 was previously identified as a multicopy suppressor of *hpr1*Δ using a different approach (Piruat and Aguilera, 1998). The N-terminus of Tho1 is homologous to the human protein hnRNP U (Aravind and Koonin, 2000). Sub2 is a putative RNA helicase involved in mRNA splicing and export. Interestingly, in a high-throughput two-hybrid analysis of the yeast genome an interaction between Sub2 and Tho1 has been observed (Ito *et al*., 2001).

The *sub2* null mutants have similar expression defects as the mutants of the THO complex (Figure 2). Thus, they cannot properly express DNA sequences such as *lacZ*. Therefore, our result provides experimental evidence that Sub2 affects transcription. Previously, it was reported that *sub2* mutants are also hyper-recombinant (Fan *et al*., 2001). Sub2, therefore, may have a function partially related to that of the THO complex. Importantly, similar to *sub2*Δ cells, *hpr1*Δ (Schneiter *et al*., 1999) and *tho2*Δ cells (P.Huertas and A.Aguilera, unpublished results; Strässer *et al*., 2002) are also defective in RNA export.

This functional link between transcription and RNA export is not only observed in THO and Sub2 mutants. We show that other mutants of the RNA export machinery such as *yra1*, *mex67* and *mtr2* also show the same patterns of hyper-recombination and transcriptional defects as the THO mutants. They do not properly express the *lacZ* sequences under the *GAL1* promoter, but show a hyper-recombination phenotype only in DNA repeats in which transcription has to proceed through sequences such as *lacZ*. Therefore, our results indicate that a defect in mRNA export in these mutants is accompanied by a defect in transcription similar to that found for mutants of the THO complex (Chávez and Aguilera, 1997; Chávez *et al*., 2001; Piruat and Aguilera, 1998). Consequently, our results suggest that THO, similar to mRNA export factors, might also act on the general process of mRNA metabolism.

Eukaryotic mRNAs are generated and processed through a number of connected metabolic steps that include transcription, capping, splicing, poly(A)$^+$ addition and RNA export (see Bentley, 1999; Minvielle-Sebastia and Keller, 1999; Hirose and Manley, 2000). Our results, indicating a transcriptional defect of RNA export mutants, provide evidence for a functional link between transcription and RNA export. Other relevant observations strengthening this conclusion are that Npl3, another essential RNA export factor, interacts with TBP, that Yra1 and Npl3 binds chromatin in a transcription-dependent manner (Lei *et al*., 2001), that ALY, the Yra1 human orthologue, has an effect in transcription activation (Bruhn *et al*., 1997) or that a blockage in RNA export causes accumulation of poly(A)$^+$ transcripts at the site of transcription (Jensen *et al*., 2001). This, together with the accumulating evidence of the physical interaction between the transcription machinery and components of the RNA processing machinery (see Hirose and Manley, 2000; Proudfoot, 2000), suggests that mRNA metabolic steps might be physically interrelated. In this context, it is worth mentioning that the Hpr1 component of the THO complex was found to be in association with the RNAPII holoenzyme (Chang *et al*., 1999). Whether this is the case for the other components of THO is still unknown. Interestingly, in a parallel study using chromatin immunoprecipitation it has been shown that Hpr1 and Tho2 are present at the 5′- and 3′-end of genes that are transcribed (Strässer *et al*., 2002).

A possible structural basis for the functional link that we observe between the four components of the THO complex (Tho2, Hpr1, Mft1 and Thp2), Sub2 and Yra1 is provided by a parallel study showing that these six proteins are found in one large protein complex termed TREX (Ho *et al*., 2002; Strässer *et al*., 2002). The similar transcription, recombination and RNA export phenotypes conferred by mutations in any of these proteins indicate that this major complex might act as a functional unit. THO represents a highly salt-resistant core complex with the strongest effect on transcription and genetic instability. Interestingly, in contrast to RNA export factors, THO is not an essential function at 30°C. It becomes essential at 37°C or when an mRNA export factor is partially deficient, as shown by the synthetic lethality of *hpr1/tho2 mex67-5* double mutants. As it has also been shown that Yra1 forms a heterodimer with Sub2 (Libri *et al*., 2001; Strässer *et al*., 2001), it is also possible that THO and Sub2–Yra1 have related but different roles in RNA metabolism. Indeed, the observation that only multicopy Sub2, but not Tho2, fully suppresses the *lacZ–URA3* expression defect of *hpr1*Δ cells, that *mft1* and *mex67-5* has a synergistic effect on recombination, and that the complex remains a structural unit in the absence of Sub2 (Figure 2C) would be consistent with this view. In any case, several results suggest that Sub2 may have a more related function with

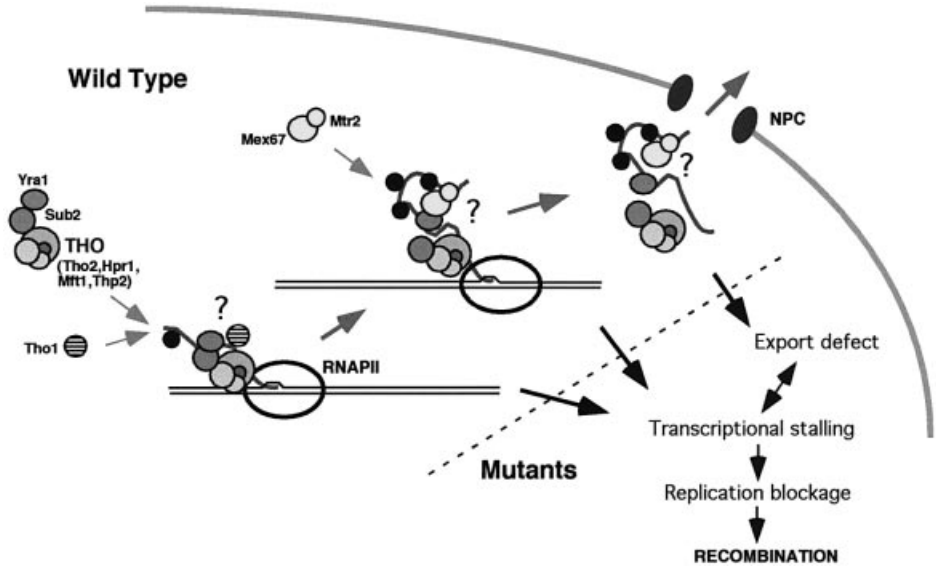

Fig. 9. Hypothetical scheme of the temporary scale of action of the THO complex and RNA export factors in relation to transcription. A question mark is shown at each step to denote our ignorance of the structural and temporary interaction between the different factors. The proteins could act on the nascent RNA sequentially as protein complexes. As a consequence of the link between transcription and RNA metabolism, a functional failure in any of these putative complexes could lead to both a defect in transcription and RNA export, regardless on their specific function. Different alternatives on how a transcriptional impairment can lead to hyper-recombination have been discussed elsewhere (see Aguilera, 2002).

the THO complex than other RNA export factors. Thus, only Sub2 is present in our highly purified His_6-tagged THO complex, but not Yra1 (Strässer *et al*., 2002). In addition, only multicopy Sub2 but not Yra1, Mex67 and Mtr2, is able to fully suppress the *lacZ–URA3* expression defect of *hpr1*Δ cells.

The functional relationship of THO with gene expression and mRNA metabolism is in agreement with our observations that THO–Sub2 can associate with RNA *in vitro* (Figure 8). In addition, the capacity of THO–Sub2 to associate with dsDNA, together with the ability of ALY, the human orthologue of Yra1, to interact with chromatin in a transcription-dependent manner (Lei *et al*., 2001) is consistent with the possibility that THO, Sub2 and Yra1 act on the nascent mRNA during transcription. The observation that Tho2 and Hpr1 are chromatin-immunoprecipitated at the 5′- and 3′-ends of transcribed genes (Strässer *et al*., 2002) could explain the transcriptional defect and the transcription-dependent hyper-recombination phenotypes of THO, Sub2 and Yra1 mutants.

The effect of Mex67/Mtr2 mutations on transcription is *a priori* surprising. Mex67/Mtr2 act at the latest step of mRNA metabolism exporting the mRNA through the nuclear pore complex (Segref *et al*., 1997; Santos-Rosa *et al*., 1998) and, in contrast to Sub2 and Yra1, a physical interaction has not been shown between THO complex and Mex67/Mtr2. The Mex67/Mtr2 heterodimer is recruited to the mRNA via Yra1 (Strässer and Hurt, 2000; Zenklusen *et al*., 2001). One possibility could be that the effect of *mex67* and *mtr2* on transcription is a consequence of an indirect effect of the mRNA export defect. However, the drastic effect of *mex67-5* on transcription activation of *GAL1–lacZ* after shifting cells to restrictive temperature (Figure 5) argues against such a possibility. Our results fit better with the view that transcription elongation and mRNA metabolism might be coupled. In this scenario, a mutation in one protein acting at the interface between transcription elongation and mRNA metabolism would affect both processes, leading to the observed defects in transcription and mRNA export. It is possible that all these proteins act sequentially (see Zenklusen and Stutz, 2001). According to the strength of the transcriptional impairment, hyper-recombination and mRNA export phenotypes of the mutants tested in this study, the THO complex would act earlier at the site of transcription and Mex67/Mtr2 later during export (Figure 9).

The transcription-dependent hyper-recombination effects of the mutants of the THO complex (*tho2*, *hpr1*, *mft1* and *thp2*) are so far the strongest evidence that THO affects transcription *in vivo* (Chávez and Aguilera, 1997; Piruat and Aguilera, 1998; Chávez *et al*., 2000). Indeed the observation that a *hpr1*Δ allele has no RNA export defect at 30°C (Schneiter *et al*., 1999), in which transcription and hyper-recombination phenotypes are detectable, argues against the possibility that RNA export is the primary defect of THO mutants. An important question arising now is how the transcriptional defect caused by mutations in genes of mRNA metabolism affect genetic stability (see Aguilera, 2002). Further biochemical and cellular analyses would help understanding of the precise role of each protein in RNA metabolism and its link to transcription and genetic instability. The existence of human homologues of Tho2, Hpr1, Sub2, Yra1, Mex67 and Mtr2, as well as the existence of a human protein complex containing Tho2, Hpr1, Sub2/UAP56 and Yra1/ALY (Strässer *et al*., 2002), indicates that this may be a conserved and relevant biological process in all eukaryotes.

Materials and methods

Strains

We used wild-type (W303-1A), *hpr1*Δ*::HIS3* (U674-1C), *tho2*Δ*::KAN* (RK2-6D) (Piruat and Aguilera, 1998), *mft1*Δ*::KAN* (WMK-2A) (Chávez

et al., 2000), *sub2*Δ (Ura⁻ segregant from DLY23) (Jensen *et al*., 2001) and *prp17*Δ (Jones *et al*., 1995) strains that have been reported previously, and isogenic derivatives obtained by genetic crosses. Mutants *yra1-1* (Strässer and Hurt, 2000), *mex67-5* (Segref *et al*., 1997) and *mtr2-26* (Santos-Rosa *et al*., 1998) were yeast strains carrying the corresponding deletion at the chromosomal loci and the mutant allele in centromeric plasmids. The *sub2*Δ strain was obtained from EUROSCARF. In addition, we constructed the WMC1-1A yeast strain carrying the *mex67-5* allele at the chromosomal locus, and derived directly from W303. Most studies on *mex67-5* mutants were carried out with this strain. All work was performed in either the W303 (R.Rothstein, Columbia University, New York, NY) or FY1679 (F.Winston, Harvard Medical School, Boston, MA) genetic backgrounds with the exception of the studies on the *sub2*Δ and *prp17*Δ mutants, which were performed in the CEN.PK and MHJ133 genetic backgrounds. In all cases we used as wild-type control the respective isogenic wild-type strain.

Plasmids

Plasmids pRS316LΔNS and pRS316L, p416-GAL1–lacZ and pSCh202 used for the studies of recombination and transcription have been published previously (Chávez *et al*., 1997; Prado *et al*., 1997). In addition, we used the plasmid YEp52T2, a 7 kb *Pst*I fragment containing *THO2* subcloned into YEp352 (P.Huertas). Plasmids YEp13-MEX67 and YEp13-MTR2 have been described previously (Segref *et al*., 1997). Plasmid YEp351-YRA1 was constructed for this study by subcloning into YEp351 (Hill *et al*., 1986) the *YRA1* ORF obtained by PCR using primers CGTATAGGATCCAATTGTTCGTT and CGTATTAAGCTTTTTT-GGTTATT. Proper functionality of the plasmid was confirmed by its capacity to complement the lethal phenotype of *yra1*Δ cells.

For the isolation of *hpr1* suppressors we constructed the plasmid pCM184-LAUR containing a *lacZ–URA3* translational fusion under the control of the *tet* promoter. For this purpose, the *lacZ–URA3* fragment was obtained by PCR from plasmid pNKY59 (Alani *et al*., 1987) using primers GCATAGCGAGGATCCATGACCATGATTACG and CTGG-CGGTAACCATCGATTTAGTTTTTGCTG, and was subcloned into the *Hpa*I site of plasmid pCM184 (Gari *et al*., 1997). As the yeast multicopy library we used MW90 constructed in YEp351 (Waldherr *et al*., 1993). As control for the study of transcription we used the plasmid pCM189-LEU2 containing the *LEU2* ORF under the *tet* promoter (S.González-Barrera and A.Aguilera, unpublished results). It was constructed by subcloning the 1.34 kb *Bam*HI–*Ssp*I *LEU2* fragment of p414GLEU2 (Piruat and Aguilera, 1998) into the *Bam*HI–*Hpa*I site of vector pCM189 (Gari *et al*., 1997).

To construct the recombination systems LI and LIr we obtained the first 800 bp of ACT1 by PCR using the primers GACGGCAGATCTATT-CTGGTATGTTCTAGCG and CCATCACCGGAATCCAAAACAAT-AC. After restriction with *Bgl*II, the resulting 500 bp fragment containing the *ACT1* intron was subcloned in both orientations into the *Bgl*II site of plasmid pRS314LB (Prado *et al*., 1997).

In vitro RNA and DNA binding assays

The His-Tho2 purified THO complex was obtained from the SChY73 strain following the procedure described previously (Chávez *et al*., 2000). *In vitro* RNA and DNA band shifting assays were performed as described by Santos-Rosa *et al*. (1998), with the exception that in the case of the THO complex the binding buffer contained 60 mM NaCl and that band-shifting was visualized in 3% non-denaturing PAGE. As RNA probe we used a 90mer RNA synthesized by T7 RNA polymerase run off transcription of the *Xba*I-linearized pBluescript SK using [^{32}P]CTP. The labelled RNA probe was purified with Sephadex G-50 after treatment with 10 U of RNase-free DNase I for 15 min at 37°C to remove contaminating DNA. As dsDNA probe we used a randomly chosen DNA region obtained by PCR and 5′-end labelled with T4 polynucleotide kinase as described previously (Chaconas *et al*., 1980). For competition assays we used as cold RNA the same 90mer SK-RNA synthesized as before but using cold instead of [^{32}P]CTPs, as cold dsDNA the same 200 bp dsDNA PCR fragment and as ssDNA a randomly chosen 60mer oligonucleotide.

THO complex purification

The THO complex was purified with either a His_6-HA-tagged Tho2 as described previously (Chávez *et al*., 2000) or by using a TAP-tagged Tho2 constructed for this study. For this, we replaced the wild-type copy of *THO2* with a *THO2–TAP* fusion obtained by PCR with primers CTTCCGCAAGGTCCCAAGGGTGGGAATTACGTCAGTA-GGTACCAGAGGTCCATGGAAAAGAGAAG and CTATCAAAGT-ACACGTTAAAATTCAGCTCGGGTATGTTAAGTACTAGTAATA-CGACTCACTATAGGG in the wild-type and *sub2*Δ strains as described previously (Rigaut *et al*., 1999). TAP-tagged purified proteins were purified essentially as described previously (Rigaut *et al*., 1999). Briefly, yeast cells were resuspended in lysis buffer [50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.15% NP-40 and a proteinase inhibitor cocktail (0.5 mM PMSF, 2 mM benzamidine, 1 μM leuptetin, 2 μM pepstatin A, 4 μM chymostatin and 2.6 μM aprotinin)] and lysed in the presence of glass beads. Lysates were clarified by ultracentrifugation at 100 000 *g* for 1 h at 4°C. The supernatant was incubated overnight at 4°C with Ig Sepharose. After washing with lysis buffer, proteins were eluted by cleavage with TEV protease. Eluted proteins were bound to cold calmodulin beads overnight. Proteins were then eluted with buffer containing 5 mM EGTA. Proteins in the various elution fractions were concentrated with TCA, resolved on a 4–20% gradient SDS–PAGE and silver-stained for visualization.

Genetic and molecular analyses

Recombination frequencies were obtained from two to three different transformants for each genotype. It was determined as the median frequency of six to 12 independent values for each transformant, as reported previously (Prado *et al*., 1997). β-galactosidase assays and northern analyses were performed according to previously published procedures (Chávez and Aguilera, 1997; Prado *et al*., 1997).

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