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Biochemical analysis of TREX complex recruitment to intronless and intron-containing yeast genes

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The TREX complex is involved in both transcription elongation and mRNA export and is recruited to nascent transcription complexes. We have examined Yra1p, Sub2p and Hpr1p recruitment to nine genes of varying lengths and transcription frequencies. All three proteins increase from the 5′ to the 3′ ends of the four intronless genes examined. A modified chromatin immunoprecipitation assay that includes an RNase step indicates that Sub2p is bound to nascent RNA, Yra1p is associated with both RNA and DNA, and Hpr1p is associated with DNA. Although Hpr1p is recruited similarly to both intronless and intron-containing genes, low Yra1p and Sub2p levels are present on a subset of intron-containing genes. The residual Yra1p and Sub2p recruitment is less RNA-associated, and this correlates with high levels of U1 SnRNP on these genes. These experiments support a model in which TREX is recruited via the transcription machinery and then Yra1p and Sub2p are transferred to the nascent RNA. On some intron-containing genes, retention and/or transfer of Yra1p and Sub2p to nascent RNA are inhibited.

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Introduction

The defining feature of the eukaryotic cell—the nuclear membrane—separates transcription and nuclear mRNA processing from most if not all translation. Within the nucleus, transcripts undergo multiple covalent processing events including the addition of a 7-methylguanosine triphosphate cap to the 5′ end, splicing to remove intervening noncoding regions, and cleavage and polyadenylation of the 3′ end. There are also noncovalent changes, which include the packaging of RNA into mRNP complexes. Some of these mRNP proteins are important for RNA export from the nucleus to the cytoplasm, including the yeast (*Saccharomyces cerevisiae*) proteins Yra1p and Sub2p (Aly/REF and UAP56 in vertebrates, respectively). It is hypothesized that Sub2p recruits Yra1p to nascent RNA (Luo *et al*, 2001; Strasser and Hurt, 2001), after which Sub2p is displaced from Yra1p by the mRNA export factor Mex67p/TAP (Strasser *et al*, 2000; Stutz *et al*, 2000; Zhou *et al*, 2000). Mex67p/TAP forms a heterodimer with Mtr2p/p15, which interacts directly with the nuclear pore to facilitate mRNP transport to the cytoplasm (Segref *et al*, 1997; Strasser *et al*, 2000).

mRNP packaging begins during transcription. An elongation complex called THO (Hpr1p, Tho2p, Mft1p, Thp2p) was biochemically purified with the mRNA export factors Sub2p and Yra1p as well as a previously unknown protein Tex1p (Chavez *et al*, 2000; Strasser *et al*, 2002). This complex was termed TREX for 'transcription/export' (Strasser *et al*, 2002), as mutations in its components show phenotypes indicative of both transcription elongation and mRNA export defects. For example, mutations in Yra1p and Sub2p are defective in transcribing G-C-rich sequences and show elevated levels of transcription-dependent recombination (Chavez *et al*, 2000; Fan *et al*, 2001; Jimeno *et al*, 2002). In addition, cells deleted for THO components are deficient in mRNA export at 37 degrees (Schneiter *et al*, 1999; Libri *et al*, 2002; Strasser *et al*, 2002).

Chromatin immunoprecipitation (ChIP) experiments show that Hpr1p, Tho2p, Sub2p and Yra1p are recruited to actively transcribed genes. All three of these proteins show similar patterns of recruitment: they increase from the 5′ end to the 3′ end of the open reading frame (ORF) and dissociate from the site of transcription coincident with cleavage and polyadenylation (Lei *et al*, 2001; Strasser *et al*, 2002; Zenklusen *et al*, 2002; Kim *et al*, 2004). However, it is unclear from the ChIP assay whether these factors are associated with DNA, perhaps via the transcription machinery, or with nascent RNA. Yra1p contains an RNA-recognition motif (RRM) suggesting that it binds to RNA. Indeed, Yra1p interacts directly with RNA *in vitro*. However, this interaction is not mediated by the RRM but rather occurs via arginine-rich regions (Rodrigues *et al*, 2001; Zenklusen *et al*, 2001). Although not directly demonstrated, Sub2p may also have RNA-binding activity, as it is predicted to be a DEAD-box helicase (Sträßer and Hurt, 2000; Kistler and Guthrie, 2001; Zhang and Green, 2001). A purified complex containing the TREX components associates with RNA *in vitro* as well as DNA (Jimeno *et al*, 2002). Moreover, it is possible that the early steps of TREX recruitment are not reflected in the steady-state protein distribution, that is, the initial recruitment of TREX could be to DNA and then some components transferred to the nascent RNA.

TREX proteins including Yra1p and Sub2p have also been implicated in splicing. Sub2p was originally identified as the yeast homolog of the metazoan splicing factor UAP56 and is required to facilitate U2 snRNP recruitment (Kistler and Guthrie, 2001; Libri *et al*, 2001; Zhang and Green, 2001). Yra1p was recently purified with yeast U2 snRNP components, suggesting that Yra1p may also have a role in splicing (Wang and Rymond, 2003). In higher eukaryotes, TREX complex components were identified in four different spliceosome purifications (Neubauer *et al*, 1998; Hartmuth *et al*,

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2002; Jurica *et al*, 2002; Rappsilber *et al*, 2002). Moreover, the Yra1p homolog Aly/REF is an integral component of the exon junction complex (EJC), which is deposited at the exon–exon junction during splicing (Le Hir *et al*, 2000). The Sub2p homolog UAP56 also associates with the EJC, but it is not an integral component of the complex (Reichert *et al*, 2002). These results suggest that Yra1p and Sub2p may act at the interface between splicing and mRNA export and could bind specifically to properly spliced mRNAs.

To learn more about recruitment of TREX components to active transcription sites, we have examined the recruitment of Yra1p, Sub2p and Hpr1p to a panel of nine different endogenous genes. By normalizing to PolII transcription, we were able to measure the levels of Yra1p, Sub2p and Hpr1p per PolII/nascent mRNA complex. Yra1p, Sub2p and Hpr1p are recruited similarly to a set of four different intronless genes. However, an assay developed to distinguish between RNA- and DNA- (transcription machinery) binding shows that these three proteins have different fates at the site of transcription. Sub2p is RNA-associated, which fits with the increase of this protein from the 5′ end to the 3′ end of genes. In contrast, the THO component Hpr1p is bound to the DNA, presumably via the transcriptional machinery. Yra1p shows yet a third pattern; the data suggest that only a fraction of the total Yra1p at the transcription site is associated with RNA. Our results suggest a two-step recruitment model, in which Yra1p and Sub2p are recruited via the TREX complex and are then transferred to the nascent RNA. Extending our analysis to five intron-containing genes indicates that Hpr1p is similarly recruited to intronless and intron-containing genes. However, Yra1p and Sub2p are poorly recruited to and retained on three out of five intron-containing genes. These and other results indicate that spliceosome assembly may interfere with the transfer of Yra1p and Sub2p to nascent RNA, suggesting a relationship between the cotranscriptional recruitment of export factors and pre-mRNA retention.

Results

TREX components increase from 5′ to 3′ on four different intronless genes

Most ChIP studies have concentrated on the recruitment of TREX components to a very limited set of genes, predominantly *PMA1* (Lei *et al*, 2001; Lei and Silver, 2002; Strasser *et al*, 2002; Zenklusen *et al*, 2002). Moreover, most studies do not control for changes in transcription rate or polymerase density, which will obviously influence the level of TREX recruitment. We have therefore extended previous analyses to include three additional intronless genes of different lengths and expression levels (Table I), and we have normalized to RNA polymerase II (PolII) recruitment. To measure simultaneously TREX and PolII recruitment from the same chromatin preparation, we used a monoclonal antibody to PolII for ChIP (8WG16). Although this reagent has been reported to show a bias against highly phosphorylated forms of PolII (Thompson *et al*, 1989), ChIP data with 8WG16 are not substantially different from those with an anti-hemagglutinin (HA) antibody and an HA-tagged RNA polymerase subunit (Komarnitsky *et al*, 2000 and Figure 1B). Indeed, ChIPs using 8WG16 indicate that PolII recruitment to the four different intronless genes largely reflects previously published differences in transcription frequency (Figure 1C and Table I).

To compare Yra1p, Sub2p and Hpr1p recruitment to this set of genes, we performed immunoprecipitations from the same chromatin preparation with antibodies specific to each epitope-tagged TREX component and with 8WG16 (see Materials and methods for details). As reported previously, Yra1p, Sub2p and Hpr1p show a very similar pattern of normalized recruitment to *PMA1*; all three proteins increase from the 5′ end to the 3′ end of the ORF (Figure 2). This same general pattern is observed on *PGK1*, *ADH3* and *FBA1*. To accommodate mRNA length differences between multiple genes, we compared TREX factor recruitment at approximately 1000 bp after transcription initiation. Yra1p and Sub2p are relatively poorly recruited to *FBA1* after approximately 1000 bp of transcription (compare striped bars, Figure 2A and B). Hpr1p is similarly recruited to *PGK1*, *ADH3* and *FBA1*, but it is more strongly recruited to *PMA1* (compare striped bars, Figure 2C). These statistically significant differences indicate that TREX components are differentially recruited to and stabilized on this set of genes.

RNA-bound proteins are detectable via ChIP

The general 5′ to 3′ increase of Yra1p, Sub2p and Hpr1p suggests that these factors may associate with the nascent transcript. To demonstrate that ChIP can detect a protein–nascent mRNA interaction, a positive control was first constructed: a protein that only interacts with nascent RNA and not at all with the transcriptional machinery. To this end, we inserted two tandem MS2 stem loops into the 5′-UTR of a *GAL1-GFP* plasmid (see Materials and methods). This

Table I Characteristics of the genes used in ChIP analysis

	ORF length (bp)	Transcriptional frequency (mRNAs/h)[a]	Intron length	Intron position
PMA1	2757	95	NA	NA
PGK1	1251	139	NA	NA
ADH3	1128	22	NA	NA
FBA1	1080	166	NA	NA
ASC1	1233	124	273	538–810
ACT1	1436	45.5	308	11–318
RPL28	961	126.3	511	50–560
DBP2	2643	61.5	1002	1274–2275
SEC27	2870	5	200	19–218

NA: not applicable.
[a]Data from Holstege *et al* (1998).

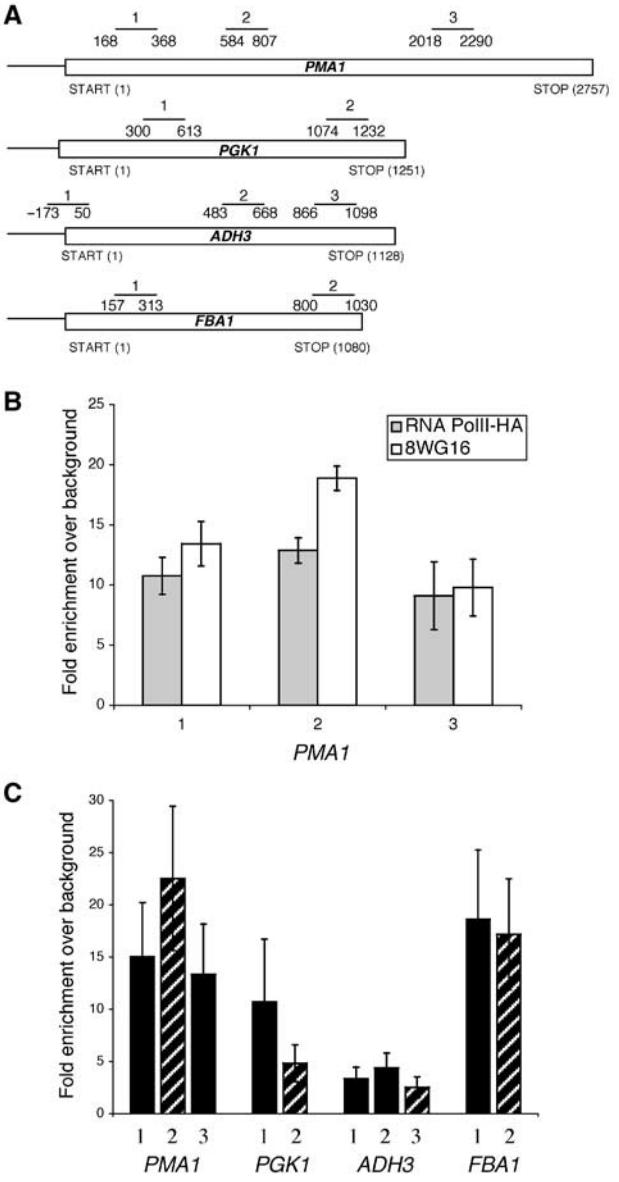

Figure 1 RNA PolII is differentially recruited to four intronless genes. (**A**) The schematic diagram shows the relative positions of the primer pairs used to analyze the recruitment to the intronless genes *PMA1*, *PGK1*, *ADH3* and *FBA1*. (**B**) The amount of PolII detected via ChIP using a monoclonal antibody against PolII (8WG16; white bars) or an anti-HA antibody to recognize an HA-tagged RNA PolII (gray bars) is very similar. The position of the *PMA1* primer pairs is indicated in (A). (**C**) The amount of PolII recruited to four different intronless genes was examined using ChIPs with the monoclonal PolII antibody (8WG16). The data are presented as the fold enrichment of PolII relative to the background binding and represent the average results from greater than 10 experiments. The striped bars indicate primer pairs that are approximately 1000 bp after transcription initiation.

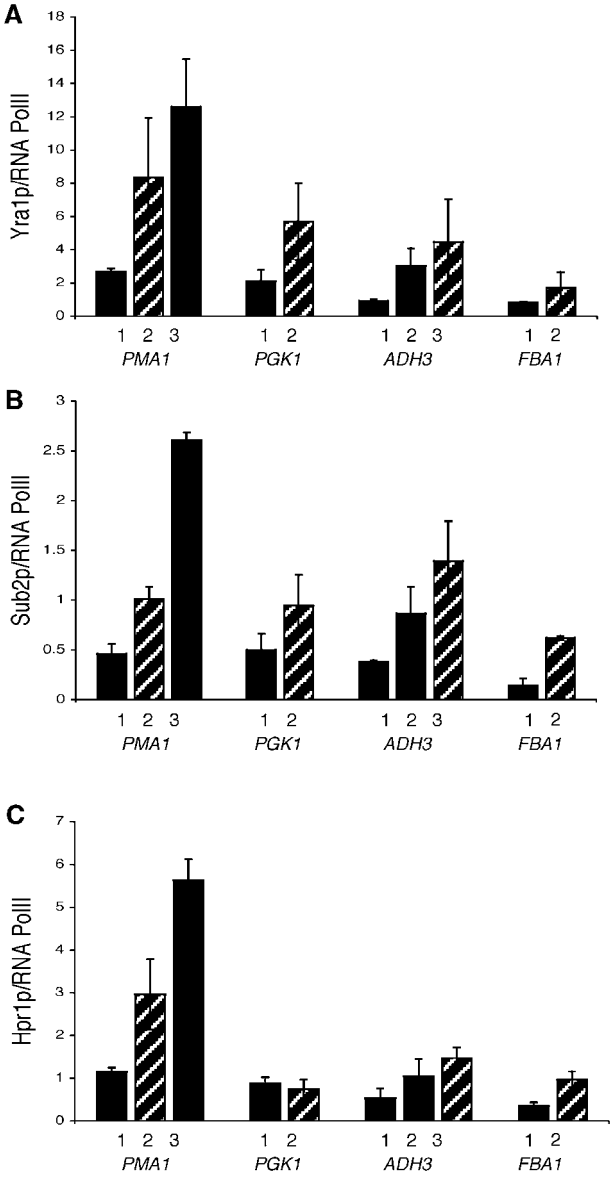

Figure 2 TREX recruitment to four different intronless genes. Single chromatin preparations from yeast strains expressing epitope-tagged Yra1p (KAY136), Sub2p (FSY1473) or Hpr1p (FSY1525) were immunoprecipitated using either antibodies recognizing PolII (8WG16) or the epitope tag. To determine the amount of TREX component recruited to the site of transcription per PolII/nascent RNA complex, the amount of TREX component recruited was divided by the amount of PolII recruited (see Materials and methods for details). The striped bars indicate which primer pairs are located approximately 1000 bp after transcription initiation. (**A**) Yra1p, (**B**) Sub2p and (**C**) Hpr1p. The data presented are the average of two independent experiments.

reporter gene was transformed into yeast along with a plasmid expressing a fusion protein containing the bacteriophage MS2 coat protein and three HA tags (MS2-HA). This system has been well characterized, even in yeast (Stripecke *et al*, 1994).

A ChIP experiment with standard anti-HA antibodies indicates that MS2-HA is recruited to *pGAL1-MS2-GFP* only when the gene is actively transcribed (Figure 3B; gray versus white bars). As an additional control, we established that there is no recruitment of MS2-HA to a *GAL1-GFP* transcript lacking MS2 stem loops, indicating that recruitment is MS2 stem loop mediated (data not shown). There is only a modest loss of signal at the 3′ end of the gene, suggesting that the nascent mRNA between the HA-tagged RNA binding protein and the 3′ DNA template remains intact and is not severely degraded.

We next modified the fixation conditions slightly and added an RNase treatment step to the ChIP protocol. (There

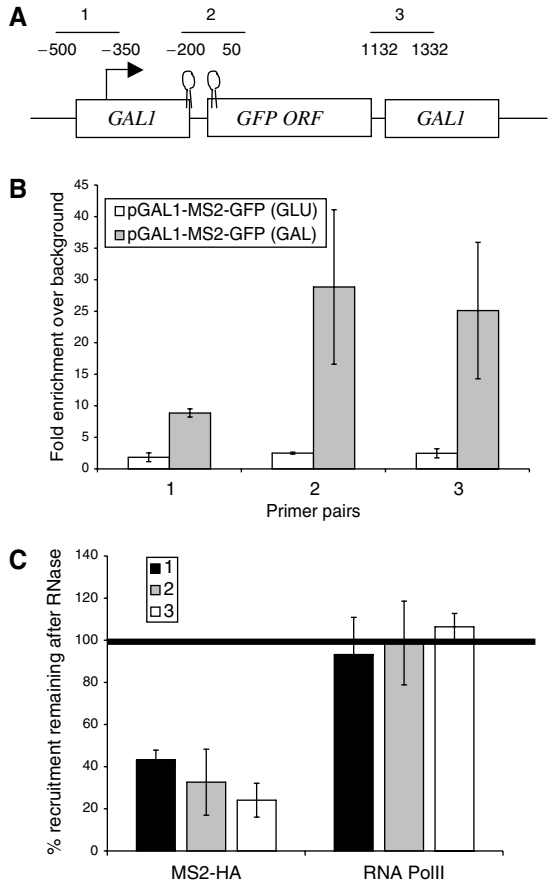

Figure 3 The recruitment of the MS2-HA fusion protein to the nascent transcription complex is sensitive to RNase. ChIPs were performed on yeast expressing an MS2-HA fusion protein and p*GAL-MS2-GFP-pA-CEN* (KAY406). (**A**) The diagram shows the features of the *GAL1-MS2-GFP-pA* construct and the position of three primer sets used in the ChIPs described in (B, C). (**B**) An MS2-HA fusion protein is cotranscriptionally recruited to a GFP mRNA containing two MS2 binding sites in the 5′-UTR (gray bars). The recruitment of MS2-HA is dependent on transcription; the MS2 fusion protein is not recruited when transcription from the *GAL1* promoter is repressed by the addition of glucose (white bars). (**C**) An RNase treatment step was added to ChIPs with antibodies against either PolII or MS2-HA to determine whether RNase can distinguish between RNA- and DNA-bound proteins in a ChIP assay. The data are presented as the percentage of protein that remains after RNase treatment (see Materials and methods). Only between 20 and 40% of the MS2-HA recruited to *GAL1-MS2-GFP* remains after RNase treatment. The data presented are the average of two independent experiments.

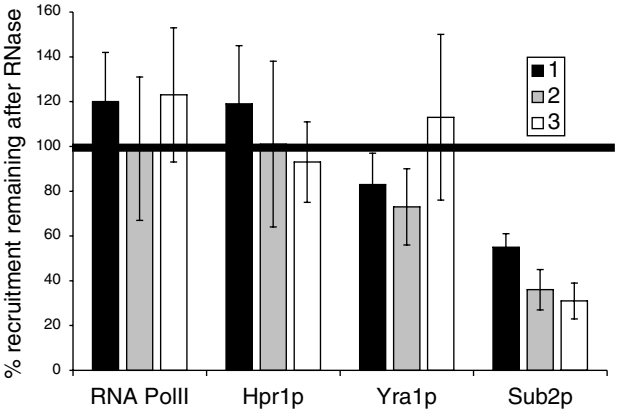

Figure 4 Sub2p, Yra1p and Hpr1p are differentially associated with the nascent mRNA. ChIPs with an RNase treatment step were performed on yeast strains expressing epitope-tagged Sub2p (FSY1473), Yra1p (KAY136) and Hpr1p (FSY1525). The methods used are identical to those described in Figure 3. The *PMA1* primer pairs used are described in Figure 1. The RNase sensitivity of Sub2p is almost identical to MS-HA; only 20–40% of the Sub2p remains at the site of transcription after RNase treatment ($n = 4$). In contrast, the RNase sensitivity of Hpr1p ($n = 4$) resembles that of PolII ($n = 10$); it is insensitive to RNase treatment. Yra1p shows partial RNase sensitivity ($n = 7$): it is more RNase sensitive than PolII at both the 5′ and middle primer; however this difference is statistically significant only at the 5′ end of *PMA1*.

is no PolII normalization used in this case; see Materials and methods for details.) Only 20–40% of the recruited MS2-HA remained after the samples were treated with RNase (Figure 3C). As expected of a protein bound to nascent RNA, the RNase sensitivity was more pronounced at the 3′ end of the gene. In contrast, the PolII signal did not decrease but actually remained constant or increased slightly with RNase treatment (Figure 3C). We often observe increases in PolII signal after RNase treatment (Figure 4), which can probably be attributed to an increase in the accessibility of the PolII epitope after digestion of nascent RNA and associated factors. This interpretation is consistent with the fact that RNase is often included in ChIP protocols to increase the signal of DNA-associated factors (Spencer *et al*, 2003). The striking difference between MS2-HA and PolII indicates that RNase sensitivity under these conditions can distinguish between RNA-bound and DNA-bound (transcription machinery-bound) factors.

The amount of Sub2p recruited to actively transcribing genes decreases upon RNase treatment

We then applied the RNase assay to the recruitment of TREX components and PolII to the *PMA1* gene. The RNase sensitivity of Sub2p recruitment is almost identical to that of the MS2-HA protein to the *GAL1-MS2-GFP* gene (compare Figure 4 with Figure 3C). This indicates that *PMA1*-bound Sub2p is predominantly nascent RNA-associated. In contrast to Sub2p, Hpr1p recruitment is essentially indistinguishable from that of PolII (Figure 4). Yra1p shows a third pattern (Figure 4). Although apparently RNase-insensitive, the difference between Yra1p and PolII RNase sensitivity is statistically significant at the 5′ (PP2) end of *PMA1* (80% for Yra1p; 120% for PolII; Figure 4). The differences between Sub2p, Yra1p, Hpr1p and PolII RNase sensitivity are not due to some unique feature of the *PMA1* gene. Similar results were obtained on *PGK1* (data not shown and Figure 7).

The RNase insensitivity of Hpr1p suggests that it is primarily DNA- or transcription machinery-associated. The RNase sensitivity of Sub2p indicates that it is primarily associated with nascent transcripts and suggests that the 5′ to 3′ increase in Sub2p protein levels is due at least in part to the increasing number of binding sites on the growing nascent mRNA. Although the RNase sensitivity of Yra1p is marginal, the experiment was repeated seven times; the data indicate that there are small but significant differences between the RNase sensitivities of Yra1p and PolII. This suggests that the Yra1p population at the site of transcription may be divided between the nascent RNA and the DNA transcription machinery.

Yra1p and Sub2p are poorly recruited to intron-containing genes

The roles of Yra1p and Sub2p in splicing and the fact that they both show an association with nascent RNA inspired a comparison of normalized TREX factor recruitment between the four intronless genes and five intron-containing genes. *ASC1*, *ACT1*, *RPL28*, *DBP2* and *SEC27* are transcribed at varying rates and contain introns of different sizes and positions (Table I). As shown for the intronless genes, the intron-containing genes recruit different amounts of PolII, which also corresponds to previous studies of transcription rates (Figure 5B and Table I).

Hpr1p is recruited similarly to eight out of the nine genes regardless of intron status (Figure 6A). The exception is the super-Hpr1p-recruiter *PMA1* (see above). Yra1p shows two different recruitment patterns on intron-containing genes: it is poorly recruited to *ASC1*, *ACT1* and *RPL28* and well recruited to *DBP2* and *SEC27* (Figure 6B). Recruitment of Yra1p to these two genes is comparable to that observed on intronless genes. The differential recruitment of Yra1p to *ACT1* and *DBP2* agrees with a previous study (Lei and Silver, 2002). Sub2p shows a very similar recruitment pattern to Yra1p with one exception: Sub2p is also poorly recruited to *DBP2* in our hands (Figure 6C), contrary to a previous report (Lei and Silver, 2002).

In an attempt to understand why Sub2p and Yra1p are differentially recruited to these intron-containing genes, we examined the recruitment of U1 snRNP component, Prp42p (Figure 6D). As shown previously, U1 snRNP is well recruited to intron-containing genes and poorly if at all to

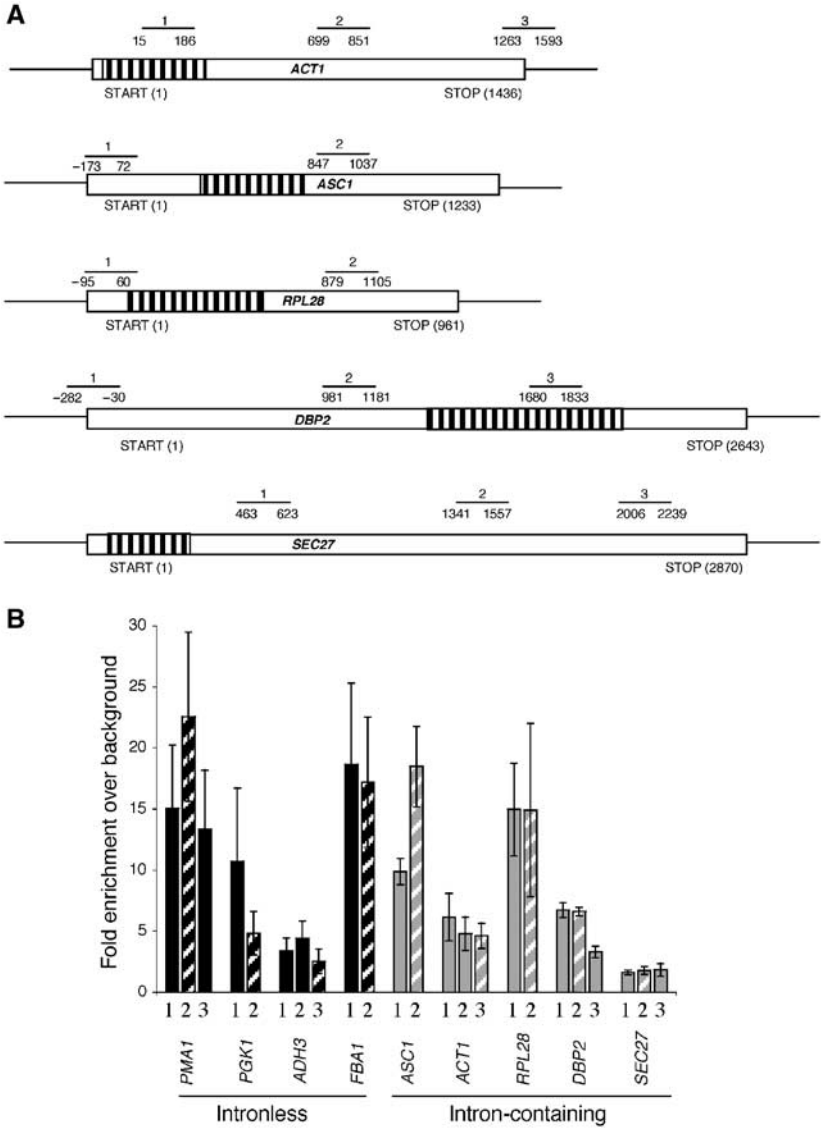

Figure 5 RNA PolII recruitment reflects the transcriptional frequency of nine intronless and intron-containing genes. (**A**) The schematic diagram shows the relative positions of the primer pairs used to examine the recruitment of PolII and TREX components to intron-containing genes. The striped regions indicate the position of the intron. (**B**) ChIPs with the monoclonal PolII antibody (8WG16) indicate that different amounts of PolII are recruited to four intronless (*PMA1*, *PGK1*, *ADH3* and *FBA1*) and five intron-containing (*ASC1*, *ACT1*, *RPL28*, *DBP2* and *SEC27*) genes. The striped bars indicate the amount of PolII recruitment approximately 1000 bp after transcription initiation. The data are presented as fold PolII enrichment relative to background binding and represent the average results from greater than 10 experiments (see Materials and methods for details).

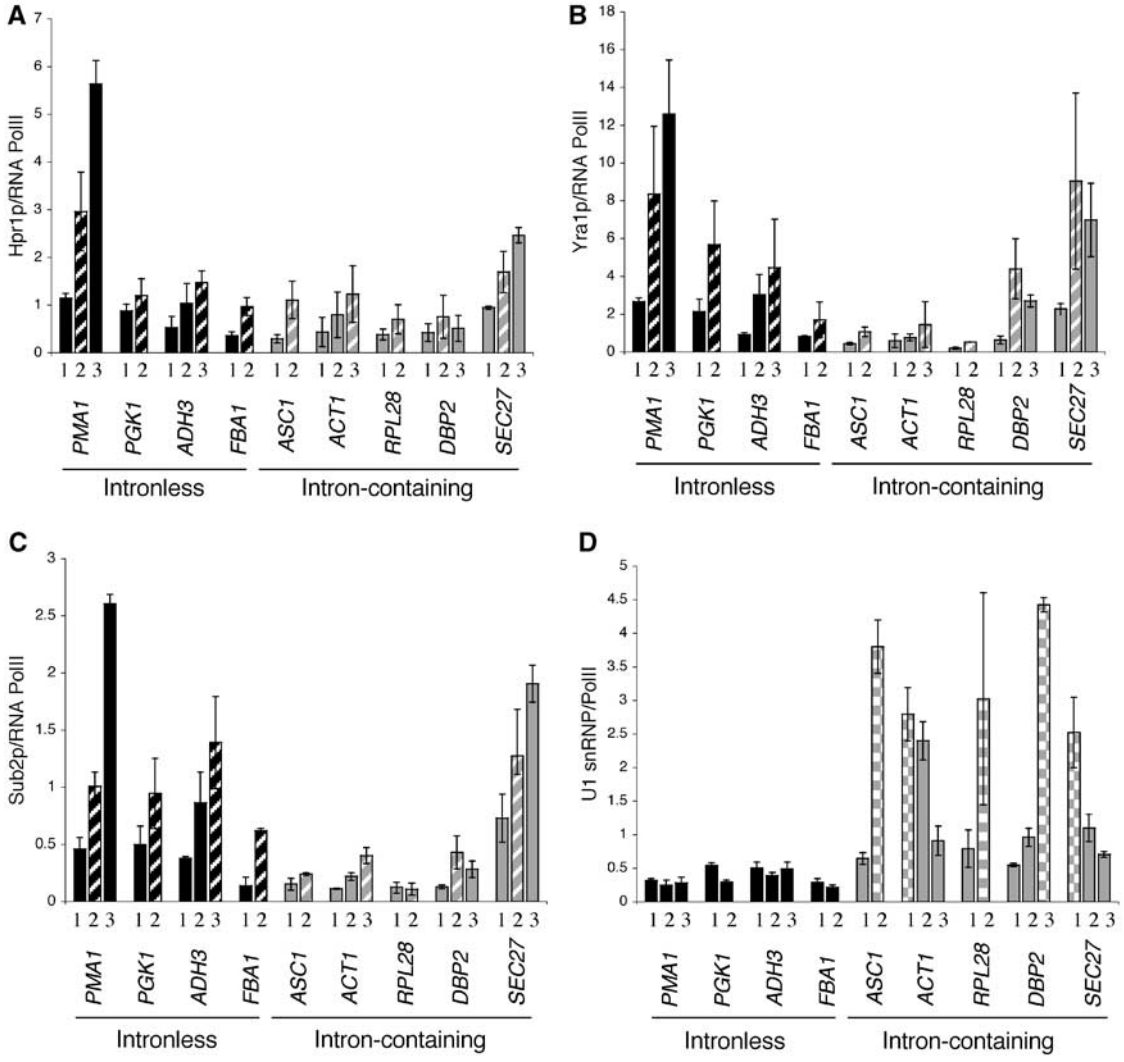

Figure 6 Yra1p and Sub2p are poorly recruited to a subset of intron-containing genes. ChIPs were performed on strains expressing epitope-tagged Yra1p (KAY136), Sub2p (FSY1473), Hpr1p (FSY1525) and the U1 SnRNP component, Prp42p (YKK25). The amount of Yra1p, Sub2p, Hpr1p and U1 snRNP recruited per PolII/nascent RNA complex is examined on five different intron-containing genes. The primer pairs used are described in Figure 5A and the striped bars (A–C) indicate primer pairs that are approximately 1000 bp after transcription initiation and the checkered bars (D) indicate the position of the intron. (**A**) Hpr1p, (**B**) Yra1p, (**C**) Sub2p and (**D**) U1 snRNP. PCR reactions for all nine genes were performed from the same ChIP samples; therefore, gene-to-gene differences in TREX and U1 snRNP recruitment cannot be attributed to experimental variation. The data presented are the average of two independent experiments.

non-intron-containing genes (Kotovic *et al*, 2003). However, only *ASC1*, *ACT1* and *RPL28* associate with U1 snRNP across their entire ORFs. (The first primer pairs of *ASC1* and *RPL28* are in the promoters.) In contrast, *DBP2* and *SEC27* have relatively low levels of U1 snRNP, before and after the introns, respectively. These results indicate that there is an inverse correlation between U1 snRNP and Sub2p/Yra1p recruitment to intron-containing genes. It suggests a model in which spliceosome assembly interferes with Yra1p and Sub2p recruitment, transfer or stabilization on some intron-containing RNAs (see Discussion).

Sub2p and Yra1p are less RNA-associated on intron-containing genes

To further address this hypothesis, we examined the RNase sensitivity of Yra1p and Sub2p on intron-containing genes (Figure 7). In contrast to the intronless genes *PMA1* and *PGK1*, Sub2p is relatively insensitive to RNase only on those intron-containing genes that show low Sub2p recruitment, for example, *ASC1* and *ACT1* (Figure 7A). Consistent with this correlation, the 3′ end of the intron-containing *SEC27* gene recruits Sub2p well and shows 'intronless' levels of Sub2p RNase sensitivity. The data suggest that low levels of Sub2p on *ASC1* and *ACT1* remain associated with the DNA or with the transcription machinery.

Owing to the marginal RNase sensitivity of Yra1p, it was not possible to detect large changes in the RNase sensitivity of Yra1p on intron-containing genes. However, there are two statistically significant differences. Yra1p shows an increased RNase insensitivity at the 5′ ends of *ACT1* and *ASC1* (Figure 7B). As these primer pairs are before or within the introns, the result suggests that Yra1p like Sub2p may not be efficiently transferred to nascent mRNA on a subset of intron-containing genes. In addition, Yra1p is more sensitive to

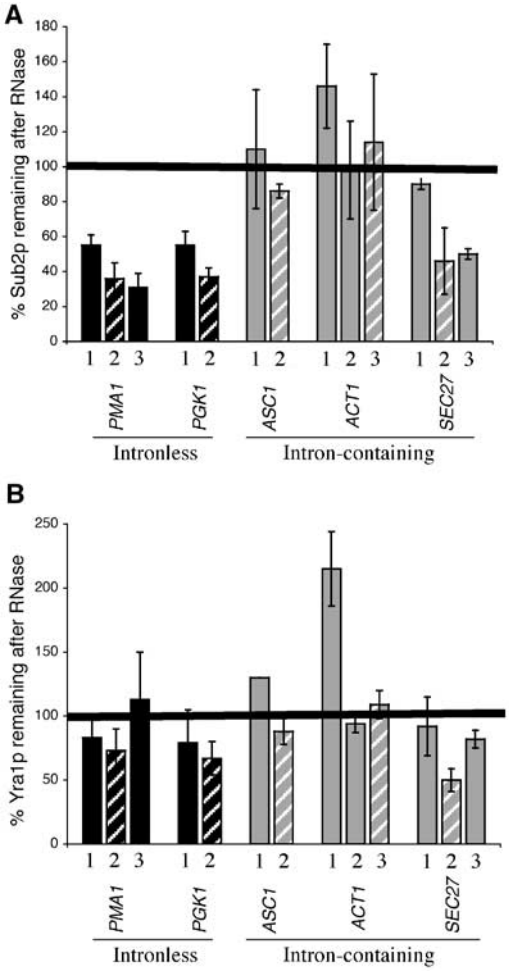

Figure 7 Poor cotranscriptional recruitment of Yra1p and Sub2p is correlated with a decrease in RNA association. Chromatin preparations from strains expressing epitope-tagged Yra1p (KAY136) or Sub2p (FSY1473) were incubated with or without RNase. The primer pairs used in these experiments are described in Figure 5A. (**A**) Sub2p and (**B**) Yra1p. The data presented are the average of two independent experiments.

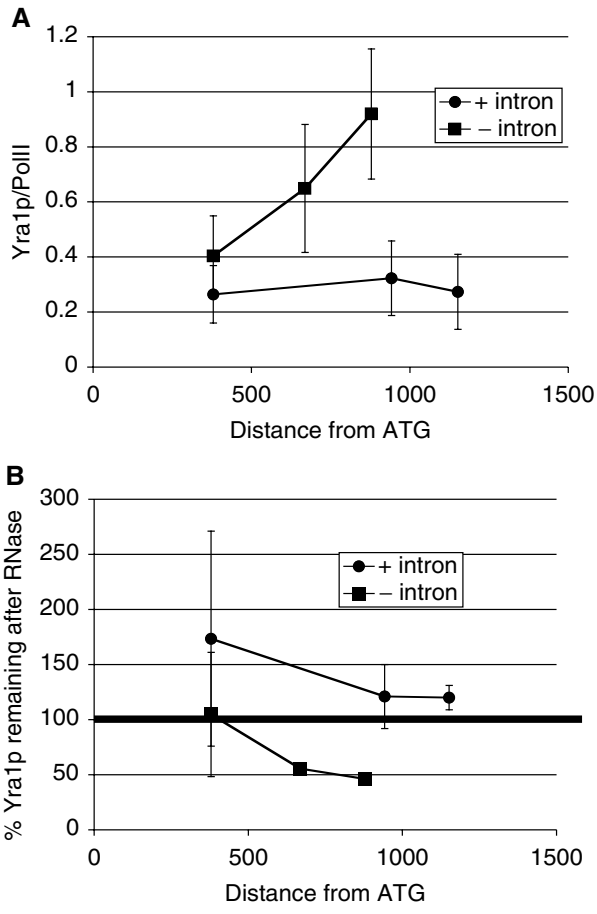

Figure 8 Removal of the intron from *ASC1* restores Yra1p levels and its RNA association. ChIPs were performed using epitope-tagged Yra1p strains expressing either *ASC1* + intron (SALY56) or *ASC1*−intron (SALY59). (**A**) The amount of Yra1p recruited to *ASC1* increases when the intron is removed; compare squares (−intron) with circles (+intron). The data are presented relative to the distance from the ATG to account for the differences in transcript length between the two *ASC1* constructs. (**B**) Yra1p becomes more RNA associated on *ASC1* when the intron is removed (compare squares (−intron) with circles (+intron).

RNase on *SEC27*, that is, an intron-containing gene that recruits high levels of Yra1p.

Yra1p recruitment increases upon intron removal

If the low Yra1p levels on a subset of intron-containing genes are due to spliceosome assembly, then intron removal should restore Yra1p recruitment and increase its RNA association. To test this hypothesis, we constructed two strains, with an intronless *ASC1* (−intron; SALY56) or a wild-type *ASC1* (+intron; SALY59) integrated at the *ASC1* genomic locus, and compared Yra1p recruitment to both *ASC1* and *PMA1*. Higher levels of Yra1p were recruited to intronless *ASC1* (Figure 8A), whereas there was no difference in Yra1p recruitment to *PMA1* in the two strains (data not shown). To account for the difference in length of the two *ASC1* transcripts, we plotted the data as a function of distance from the ATG. A similar but smaller increase in Yra1p recruitment is observed when the intron is removed from *ACT1* (data not shown; see Discussion). As predicted, the increase in Yra1p recruitment on intronless *ASC1* was accompanied by an increase in RNase sensitivity (Figure 9B). This suggests that the presence of an intron in *ASC1* inhibits Yra1p transfer to the nascent RNA.

Discussion

We have examined the cotranscriptional recruitment of three TREX proteins to nine different yeast genes. Yra1p, Sub2p and Hpr1p all show a striking 5′ to 3′ increase in recruitment on almost all these genes. However, an RNase assay distinguishes between the three proteins. Only Sub2p appears cleanly bound to nascent RNA, with no evidence for an association with DNA or with the transcription machinery. Hpr1p, in contrast, is directly or indirectly bound to DNA. Another THO complex component, Tho2p, shows the same complete RNase insensitivity as Hpr1p (data not shown). Yra1p is in a third category and apparently contacts DNA as well as the nascent RNA. The data suggest that Yra1p and Sub2p are recruited to the site of transcription as part of the TREX complex and then Sub2p and to a lesser extent Yra1p are transferred to the nascent RNA. Hpr1p is recruited

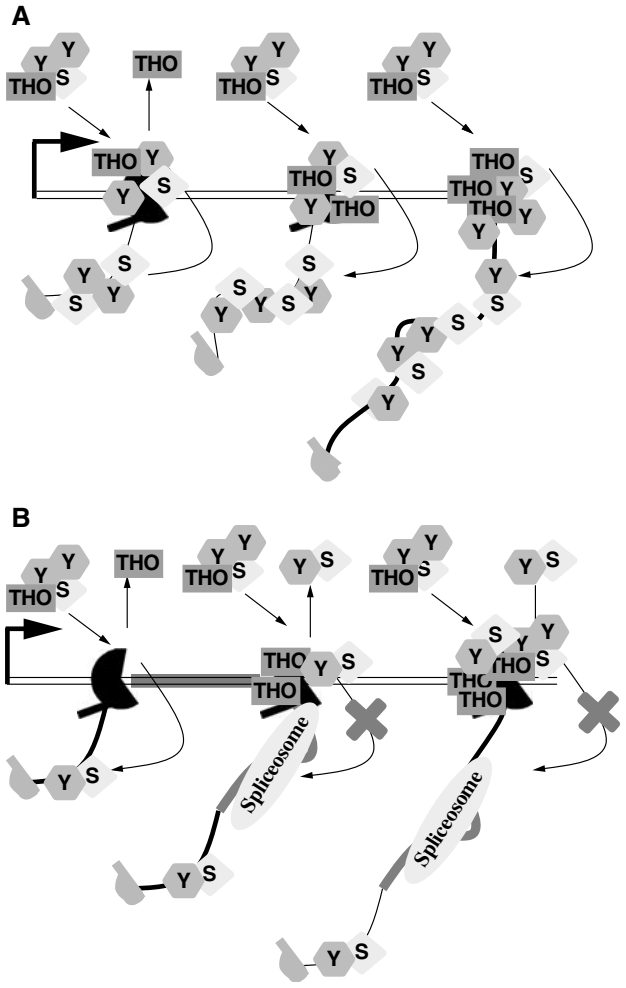

Figure 9 Model for TREX component recruitment to intronless and intron-containing genes. (**A**) Our data indicate that once the TREX complex is recruited to the site of transcription, presumably via the transcription machinery, the complex dissociates. The THO complex remains associated with the DNA or transcription machinery. Sub2p is immediately and efficiently transferred to the nascent RNA. The Yra1p divides into two pools: one remains associated with the DNA and the other is transferred to the RNA. (**B**) On intron-containing genes, the TREX complex is recruited normally but the transfer of Sub2p and Yra1p to the RNA may be hindered by the spliceosome. As a result, less Sub2p and Yra1p are retained at the site of transcription (S = Sub2p; Y = Yra1p).

similarly to both intronless and intron-containing genes. In contrast, both Yra1p and Sub2p accumulate poorly on a substantial subset of intron-containing genes. Taken together, we suggest that successful transfer of Yra1p and Sub2p from the transcription machinery to the nascent RNA may be a critical step in linking transcription, splicing and export (Figure 9).

The simplest explanation for the striking 5′ to 3′ enrichment of all TREX complex components is that the entire complex is recruited by or transferred to the nascent RNA. This possibility is consistent with the fact that Yra1p, Sub2p and Hpr1p all dissociate from the site of transcription after polyadenylation has occurred (Kim *et al*, 2004). A problem, however, is the striking distinction between the RNase sensitivity of Sub2p and Hpr1p. Moreover, Sub2p relies on Hpr1p for nascent recruitment, that is, Sub2p recruitment to *PMA1* is substantially decreased in an *hrp1*Δ strain both with and without normalization to PolII (KC Abruzzi and M Rosbash, preliminary results; Zenklusen *et al*, 2002, respectively). This suggests that Sub2p is recruited via the TREX complex to DNA or to the transcription machinery and is then transferred to the nascent RNA. An equally plausible possibility is that TREX is recruited to nascent mRNA and Hpr1p is subsequently transferred to the DNA. However, we would then expect Hpr1p to be decreased on the intron-containing genes. The data argue in favor of the 'DNA and then transfer to RNA' interpretation.

The 5′ to 3′ increase in Sub2p signal is probably due to the longer nascent RNAs associated with the 3′ ends of genes. However, the same increase in the recruitment of Yra1p and especially Hpr1p requires another explanation, as it is different from other nascent proteins such as transcription and polyadenylation factors (Licatalosi *et al*, 2002; Ahn *et al*, 2004; Kim *et al*, 2004). Although changes in epitope availability cannot be excluded, there is probably more Yra1p, Hpr1p and other THO components at the 3′ end of genes, or they bind more tightly in these regions (lower off-rate). Many changes occur as polymerase transcribes across a gene, including changes in PolII phosphorylation (Komarnitsky *et al*, 2000), the recruitment of factors important for 3′-end formation (Licatalosi *et al*, 2002; Ahn *et al*, 2004; Kim *et al*, 2004) and DNA supercoiling (Huertas and Aguilera, 2003). It is possible that one or more of these 3′-end events could stimulate TREX binding or retention.

The complete transfer of Sub2p to RNA is consistent with an analysis of giant Balbiani ring nascent mRNPs, namely, that the Sub2p ortholog coats the entire mRNP uniformly (Kiesler *et al*, 2002). The Yra1p ortholog has a different distribution (Kiesler *et al*, 2002), consistent with the differences we observe in RNase sensitivity. Nonetheless, Yra1p may also be completely transferred to the nascent RNA. The intermediate RNase sensitivity may reflect some residual affinity for the transcription machinery, which is captured during the *in vivo* crosslinking procedure. Another possibility is that Yra1p is incompletely transferred to the RNA, leaving some protein bound to DNA or to the transcription machinery. This might indicate that there is less Yra1p than Sub2p bound to nascent RNA. Alternatively, Yra1p and Sub2p could still be stochiometric on nascent RNA, which implies a prior suprastochiometric recruitment of Yra1p to the transcription machinery. This could be due either to a suprastochiometric association of Yra1p relative to Sub2p within TREX or to an additional mode of Yra1p recruitment, followed by stochiometric and coupled transfer of Sub2p and Yra1p to nascent RNA (Figure 9A). Indeed, coupled transfer accommodates the known association of Sub2p with Yra1p, and the similarity in Sub2p and Yra1p recruitment is striking—their differences in RNase sensitivity notwithstanding.

Another example of Yra1p and Sub2p co-regulation is the low levels of Yra1p and Sub2p on *ACT1*, *ASC1* and *RPL28*. These intron-containing genes are expressed at different rates, range between 1000 and 1400 bp in length, and contain introns either at the 5′ end or middle of the genes. As this effect is paralleled by lower RNase sensitivity and high U1 snRNP recruitment, it suggests that cotranscriptional spliceosome assembly inhibits transfer to, or retention on, nascent pre-mRNA (Figure 9B). This may be due to passive occlusion (the spliceosome occupies pre-mRNA sites that would other-

wise receive Sub2p and Yra1p), or it may reflect some more active inhibition of transfer. These models are supported by results showing that the removal of the *ASC1* intron increases Yra1p recruitment and Yra1p association with the nascent RNA (Figure 8).

Despite the increase in Yra1p recruitment to the *ASC1* cDNA, our results suggest that the inhibition of Yra1p and Sub2p recruitment may be complicated. When the intron is removed from *ACT1*, the increase in Yra1p recruitment is less striking (data not shown). Similar results are observed for Sub2p recruitment to the *ASC1* cDNA; Sub2p increases only marginally and the differences are not significant (data not shown), suggesting that the splicing inhibition of Yra1p and Sub2p recruitment and transfer is gene specific and complicated. This may be related to the diverse effects of splicing on transcription and polyadenylation in yeast as well as metazoa (Brinster *et al*, 1988; Niwa *et al*, 1990; Palmiter *et al*, 1991; Dye and Proudfoot, 1999; Lacy-Hulbert *et al*, 2001; Furger *et al*, 2002).

In contrast to *ASC1*, *ACT1* and *RPL28*, the *SEC27* and *DBP2* intron-containing genes recruit and retain high levels of Yra1p and/or Sub2p. Our data suggest that the nascent transcripts of these long and unusual intron-containing genes contain substantial stretches of RNA free of spliceosomes. Although U1 snRNP recruitment is robust in the region adjacent to the 5′-intron of *SEC27*, U1 snRNP is undetectable in the middle and 3′ end of *SEC27*. Consistent with this observation, Sub2p is less associated with the RNA only at the 5′ end of SEC27. In the case of *DBP2*, no U1 snRNP is recruited prior to the 3′-intron. This suggests that *DBP2* resembles an intronless gene until the intron emerges 1273 bp after transcription initiation and that Yra1p binds normally prior to intron emergence. Previously published experiments showed that mutations in early and late splicing factors decrease and increase Yra1p recruitment on *DBP2*, respectively (Lei and Silver, 2002). It is difficult to interpret these results in the light of our U1 snRNP data, but perhaps they are due to complicated effects of splicing on transcriptional recruitment.

The low levels of Sub2p and Yra1p on some intron-containing genes was surprising, given the relationship of these proteins to splicing (e.g., Kistler and Guthrie, 2001; Libri *et al*, 2001; Zhang and Green, 2001). Although our data do not preclude a role in splicing, they do argue against the presence of an exon junction complex (EJC) in yeast, at least as a major means of recruiting Yra1 and Sub2p to most intron-containing genes. The earlier study examining the recruitment of Yra1p and Sub2p to *DBP2* and *ACT1* explained the low recruitment of Yra1p and Sub2p to *ACT1* by invoking an EJC-like mechanism, that is, by proposing that Yra1p requires a long 5′-exon to bind to intron-containing RNAs (Lei and Silver, 2002). However, our data show that 5′-exon length does not correlate positively with Yra1p and Sub2p recruitment: Yra1p and Sub2p are poorly recruited to the long 5′-exon-containing gene *ASC1* and well recruited to the short 5′-exon-containing gene *SEC27*. Given the features of the five intron-containing genes we have examined, we suspect that low recruitment will be the more common result for the entire set of yeast intron-containing genes.

Most unspliced pre-mRNAs are poorly exported to the cytoplasm in yeast (Legrain and Rosbash, 1989; Rutz and Seraphin, 2000). A recent study indicates that nuclear Mlp1p may be a central player in this retention process: it recognizes U1 snRNP-bound pre-mRNAs at the nuclear periphery and helps prevent their export (Galy *et al*, 2004). Our data suggest that yeast may have a second line of defense against the nuclear export of pre-mRNAs. mRNP export factors may not be adequately loaded onto pre-mRNA during early spliceosome formation. This may inhibit their efficient export from the nucleus.

Materials and methods

Strains and media

All yeast strains used in this study are from the W303 background and are derivatives of Y368 except where noted (Table II). We used standard methods for yeast manipulations (Guthrie and Fink, 1991).

Plasmid and strain construction

To construct a *LEU2*-marked plasmid expressing *YRA1* with a triple HA epitope tag, pFS2233 (Zenklusen *et al*, 2001) was cut with *Bam*H1 and the resulting fragment was ligated into *Bam*H1-digested pRS315 (Sikorski and Hieter, 1989) to produce pKA14. To construct p*GAL1-TRP1*-2μ, the *GAL1* locus was PCR amplified using overlap PCR to replace the *GAL1* ORF with *Bam*HI/*Sal*I cloning sites. This PCR product was cloned using *Bgl*II/*Xho*I into *Bam*HI/*Sal*I of *TRP1*-2μ (Libri *et al*, 2002). To construct p*GAL1-GFP-TRP1*-2μ, the green fluorescent protein (GFP) was PCR amplified from plasmid pJK19-1 (a gift from P Silver) and cloned as a *Bam*HI/*Sal*I fragment into *GAL1-TRP1*-2μ. To construct p*GAL1-(MS2)-GFP-pA-TRP1-CEN*, the *GAL1* promoter, GFP ORF, and *GAL1* 3′ region were PCR amplified from p*GAL1-GFP-pA-TRP1-2*μ using overlap PCR to introduce two MS2 stem loops (CGTACACCATCAGGGTACG CGAGCTAGCC CATCGCGTACACCATCAGGGTACG) into the *GAL1* 5′ untranslated region. This PCR product was cloned using *Bgl*II/*Xho*I into *Bam*HI/*Sal*I of pRS314. The MS2 stem loop sequence was described Jurica *et al* (2002). *TDH3-LEU2-CEN* was constructed by PCR amplifying the *TDH3* locus using overlap PCR in which *Bam*HI/*Sal*I cloning sites replace the *TDH3* ORF. This PCR product was cloned using *Bgl*II/*Xho*I into *Bam*HI/*Sal*I of pRS315. To construct p*MS2-HA-LEU2-CEN*, the bacteriophage MS2 coat protein ORF was PCR amplified from MS2-MBP (a gift from M Jurica and M Moore; Jurica *et al*, 2002) to introduce sequences encoding an N-terminal SV40 nuclear localization signal and three C-terminal HA tags. This PCR product was cloned using *Bam*HI/*Xho*I into *Bam*HI/*Sal*I of p*TDH3-LEU2-CEN*. To construct p*ASC1-Bam*HI-*Sal*I-*ASC1*, the *ASC1* locus was PCR amplified using overlap PCR to replace the *ASC1* ORF with *Bam*HI/*Sal*I cloning sites. This PCR product was digested with *Bgl*II/*Xho*I and ligated into *Bam*HI/*Sal*I-digested pRS426 to create p*ASC1-Bam*HI-*Sal*I-*ASC1*. The *ASC1* ORFs, with or without the intron, were amplified from plasmids pASC9 and pD16 (gifts from N Proudfoot; Furger *et al*, 2002), respectively. The resulting PCR products were digested with *Bam*HI/*Sal*I and ligated into p*ASC1-Bam*HI-*Sal*I-*ASC1* to generate pSAL1 (+intron) and pSAL2 (−intron). pSAL1 and pSAL2 were linearized using *Ahd*I, amplified with primers SALO48 and SALO49, and the resulting product was transformed into yeast (KAY136) to generate SALY56 and SALY59.

Chromatin immunoprecipitations

ChIPs were performed using methods previously described (Komarnitsky *et al*, 2000) with the following exceptions. The crosslinking time was reduced from 20 to 5 min when RNase treatment was performed. Yeast cells were lysed using a Mini-Bead Beater 8 (BioSpec Products). All centrifugation steps were performed in a refrigerated microcentrifuge at maximum speed. Samples were sonicated for six cycles of a 20 s pulse followed by a 20 s pause using a 550 Sonic Dismembranator (Fisher Scientific). This sonication treatment is sufficient to shear the chromatin to an average size of between 500 and 1000 bp. Monoclonal antibodies against total RNA PolII (8WG16) were purchased from Covance. The anti-HA monoclonal (12CA5) was purchased from Roche. Each of these antibodies was preincubated with protein A–Sepharose 4B beads (Zymed). For protein-A-tagged factors, IgG Sepharose beads (Amersham Pharmacia) were used.

The amount of DNA in the 'input' and 'IP' samples was quantitated using real-time PCR (Rotorgene by Corbett Research).

Table II Strains and plasmids

Strain/plasmid	Genotype	References
Strains		
FSY1473	MATa, *ura3, lys2, trp1, ade, his3, leu2, sub2*::*HIS3* plus pFS2625	Zenklusen *et al* (2002)
FSY1525	MATa, *ura3, lys2, trp1, his3, ade, leu2, sub2*::*HIS3, Hpr1-ProtA* plus *pSUB2-URA3-CEN*	Zenklusen *et al* (2002)
KAY136	MATa, *yra1*::*HIS3*, *ade2, his3, leu2, trp1, ura3* plus pKA14	This study
KAY406	FY368 plus p*MS2-HA-LEU2-CEN* & p*GAL-MS2-GFP-TRP1-CEN*	This study
YKK25	MATa, *ura3, leu2, trp1, his3, can1-100, psi ADE+ GAL+ prp42*::*HA-PRP42-KanR*	Kotovic *et al* (2003)
SALY56	MATa, *yra1*::*HIS3*, *ASC1*::*ASC1-URA3*, *ade2, his3, leu2, trp1, ura3* plus pKA14	This study
SALY59	MATa, *yra1*::*HIS3*, *ASC1*::*ASC1-intron-URA3*, *ade2, his3, leu2, trp1, ura3* plus pKA14	This study
YSB749	MATa, *ura3-52, leu2-3, 112, trp1-1, his3200, ade2-1, RPB3-(HA)3*::*LEU2*	Komarnitsky *et al* (2000)
Plasmids		
pASC9	*ASC1-URA3-2*μ	Furger *et al* (2002)
pD16	*ASC1-intron-URA3-2*μ	Furger *et al* (2002)
pFS2625	*SUB2-PROTA-LEU2-CEN*	Zenklusen *et al* (2002)
pFS2233	*HA-YRA1*(+intron)*-TRP1-CEN*	Zenklusen *et al* (2001)
pKA14	*HA-YRA1*(+intron)*-LEU2-CEN*	This study
pSAL1	*ASC1pro-ASC1-ASC1-3′UTR-URA3-2*μ	This study
pSAL2	*ASC1pro-ASC1-intron-ASC1-3′UTR-URA3-2*μ	This study
	*ASC1pro-BamHI-SalI-ASC1-3′UTR-URA3-2*μ	This study
	*TRP1-2*μ	Libri *et al* (2002)
	*GAL1-TRP1-2*μ	This study
	GAL1-(MS2)-GFP-pA-TRP1-CEN	This study
	TDH3-LEU2-CEN	This study
	MS2-HA-LEU2-CEN	This study

To verify that the real-time PCR machine gave accurate and reliable results, we did a side-by-side comparison of real-time PCR and radioactive PCR followed by phosphor imaging. We found that these two techniques gave nearly identical results (data not shown). The real-time PCR reactions contained 0.5 U of platinum *Taq* polymerase (Invitrogen), 0.1 mM dNTP mixture (Invitrogen), 0.25 μM each primer, 1.5 mM $MgCl_2$ and 1 × platinum *Taq* PCR buffer (20 mM Tris–HCl (pH 8.4), 500 mM KCl) and 0.2 × concentration of Sybr Green (Molecular Probes). PCR was performed under the following conditions: 90 s at 95°C followed by 45 cycles of 10 s at 94°C, 30 s at 55°C and 45 s at 72°C. Each 'input' and 'IP' sample was analyzed in triplicate using real-time PCR, and the resulting values were averaged to determine the concentration of each sample. The primers used in this study were chosen using Primer3 from the Whitehead Institute (Rozen and Skaletsky, 2000) and are described in Figures 1A and 5A, and Supplementary Table I. The *PMA1* and intergenic primers (Intergenic V-1 and V-2) were previously described (Komarnitsky *et al*, 2000).

The calculations and normalizations for each ChIP were carried out as previously described (Komarnitsky *et al*, 2000; Ahn *et al*, 2004; Kim *et al*, 2004). Briefly, the enrichment of the protein of interest (i.e., Yra1p) above background was calculated by dividing (IP signal for gene-specific primer pair/IP signal for intergenic primer pair) by the (input signal for gene-specific primer pair/input signal for intergenic primer pair). The 'intergenic' primer pair is in a nontranscribed region of chromosome five and should account for any nonspecific DNA binding. A fold enrichment of 1 indicates that the amount of DNA immunoprecipitated by the protein of interest is no higher than would be expected from nonspecific DNA binding.

Protein enrichment was normalized to PolII recruitment to account for differences in transcription frequency both across genes and between different genes. For these experiments, immunoprecipitations of the factor of interest (i.e., Yra1p) and PolII were preformed from the same chromatin preparation. The fold enrichment over background was determined individually for each protein (see description above), and the fold enrichment for the factor of interest (i.e., Yra1p) was divided by the fold enrichment for RNA PolII (i.e., Yra1p/PolII). When comparing the recruitment of TREX components on nine different genes (Figure 6), all of the PCR was performed from the same sample. Therefore, the gene-to-gene differences in TREX component and U1 snRNP recruitment cannot be attributed to experimental variation.

When an RNase treatment step was added to the ChIP protocol, chromatin from the same experiment was treated with either 7.5 U of RNase A and 300 U of RNase T1 (RNase A/T1 Cocktail; Ambion) or an equivalent volume of RNase storage buffer (10 mM Hepes pH 7.2, 20 mM NaCl, 0.1% Triton X-100, 1 mM EDTA and 50% glycerol v/v). After incubating at room temperature for 30 min, immunoprecipitations were performed as described above. To calculate the 'percentage of factor remaining after RNase treatment', we divided the fold enrichment for the RNase-treated sample by the fold-enrichment for the non-RNase-treated sample and multiplied the result by 100. We did not normalize to PolII in the RNase experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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