Defects in mRNA 3'-end formation, transcription initiation, and mRNA transport associated with the yeast mutation *prp20*: possible coupling of mRNA processing and chromatin structure

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A temperature-sensitive lethal mutation in *Saccharomyces cerevisiae*, *prp20-1*, causes defects in several different steps in mRNA metabolism, including mRNA 3'-end formation, transcription initiation, and mRNA transport. Previous work has demonstrated that *prp20* mutants are defective in actin pre-mRNA splicing. *PRP20* is related, both in structure and function, to the *RCC1* gene of mammals and the *PIM1* gene of *Schizosaccharomyces pombe*, both of which appear to regulate entry into mitosis and chromosome condensation. In this report we demonstrate that, after a shift of *prp20* mutants to the restrictive temperature, transcripts of several genes (*CUP1*, *CYH2*, and *GAL10*) are produced that extend 1–10 kb beyond their normal polyadenylation sites. The failure in 3'-end formation occurs within 1–2 min of the temperature shift. Transcription initiation also is disrupted, in that initiation sites upstream of the normal cap site are used. mRNA transport from nucleus to cytoplasm also is perturbed: In situ hybridization using an oligo(dT) probe demonstrates accumulation of poly(A) in the nucleus, consistent with the accumulation of longer bulk poly(A) (up to ~90–100 nucleotides) and with a failure to transport newly synthesized RNA to the cytoplasm. We demonstrate that *prp20* and *rna1* mutants are very similar, if not identical, with respect to each of these biochemical phenotypes. In light of the putative role of *PRP20* in mitotic control, our results suggest a common step in that process and multiple steps in mRNA synthesis and maturation. We speculate that the perturbations in mRNA processing are the result of effects on the chromatin–nascent RNP–transcription complex or misregulation of a cell cycle component that modifies multiple mRNA-processing activities.

[Key Words: mRNA processing; *RCC1*; *PIM1*; *RNA1*; polyadenylation; nuclear structure; chromatin]

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mRNAs formed in eukaryotic cells are subject to many post-transcriptional processing steps, any of which can regulate the quantity or type of mRNA produced. These steps include capping, splicing, 3'-end cleavage, poly(A) addition, transport from nucleus to cytoplasm, poly(A) removal, and destruction of the mRNA. Their biochemical mechanisms appear to be similar in yeast and in mammals in those cases in which it has been examined (e.g., see Green 1991; Guthrie 1991). Here, we focus on post-transcriptional processing in *Saccharomyces cerevisiae*, concentrating on the role of the *PRP20* gene product in mRNA 3'-end formation and transport.

The mechanisms of mRNA 3'-end formation in yeast and mammals are similar, but distinct. In both yeast and mammals, formation of mRNA 3' termini involves endonucleolytic cleavage of an extended primary transcript. This is followed by the post-transcriptional addition of a poly(A) tract, containing 60–100 nucleotides in yeast (Groner et al. 1974) and 250–300 nucleotides in mammals (for review, see Wickens 1990). The enzyme that adds the poly(A) tail, poly(A) polymerase, is very similar in sequence in yeast and mammals (Lingner et al. 1991; Raabe et al. 1991).

Biochemical analysis of mutations that disrupt mRNA processing in yeast has provided invaluable insight into the mechanism of RNA splicing. Some 20 genes (termed *PRP*, for pre-mRNA processing) have been identified that are likely to be essential for this one processing step. For

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3'-end formation, however, genetic studies thus far have revealed only a single gene that is essential (Lingner et al. 1991). This gene encodes the poly(A) polymerase.

Mutations in two other yeast genes affect multiple post-transcriptional processing events, including mRNA 3'-end formation. A mutation in the *PRP20* gene (also called *SRM1*) has been reported to cause accumulation of longer mRNA species, presumably by a failure to terminate or process 3' ends (Aebi et al. 1990). This mutation also affects actin-intron splicing (Vijayraghavan et al. 1989), and nuclear morphology (Aebi et al. 1990) and suppresses the mating defect in a strain lacking mating-factor receptor (Clark and Sprague 1989). Similarly, mutations in the yeast *RNA1* gene not only disrupt mRNA 3'-end formation but also stable RNA production (Hutchison et al. 1969), tRNA splicing (Hopper et al. 1978; Knapp et al. 1978), and rRNA processing (Hopper et al. 1978) and may affect mRNA transport from the nucleus as well (Hutchison et al. 1969; Shiokawa and Pogo 1974; Piper and Aamand 1989). It is not clear which, if any, of the array of phenotypes associated with the *prp20* and *rna1* mutations correspond to the primary sites of action of the proteins.

PRP20 is a member of a gene family that includes *Schizosaccharomyces pombe* *PIM1*, mammalian *RCC1*, and related genes in *Xenopus* and *Drosophila* (Ohtsubo et al. 1987; Aebi et al. 1990; Nishitani et al. 1990; Frasch 1991; Matsumoto and Beach 1991). These genes are not only similar in structure but in function as well. By genetic criteria, mammalian *RCC1* and *S. pombe* *PIM1* are required for control of chromosome condensation: Loss-of-function mutations in these genes cause interphase chromosomes to undergo mitotic condensation prematurely (Ohtsubo et al. 1987; Matsumoto and Beach 1991). In *S. cerevisiae*, *prp20* mutants display altered nuclear morphology, consistent with a defect in chromatin structure (Aebi et al. 1990). The chromosome condensation defect may be caused by an increased *CDC2* activity and reflect premature entry into mitosis (Matsumoto and Beach 1991). Because the mammalian *RCC1* gene and the yeast *PRP20* gene complement one another, they are very likely to have overlapping functions in vivo (Clark et al. 1991; Fleischmann et al. 1991; Ohtsubo et al. 1991).

Although the biochemical activities of these proteins in vivo has not been demonstrated, the *RCC1* protein has been shown in vitro to bind to chromatin and DNA (Ohtsubo et al. 1989) and to stimulate the GDP-GTP exchange activity of the *ras*-like nuclear protein *RAN* (Bischoff and Ponstingl 1991). Like *PRP20* protein, the *RCC1* protein is located in the nucleus (Ohtsubo et al. 1989; Fleischmann et al. 1991).

Here, we characterize in more detail the defects in 3'-end formation and poly(A) metabolism in *prp20* mutant strains, comparing them to those observed in *rna1* mutants. We show that *prp20* mutants exhibit defects in 3'-end cleavage on several different mRNAs, failing to process RNAs at their normal polyadenylation sites. In addition, *prp20* mutants initiate transcription at abnormal sites. By in situ hybridization, we demonstrate that

the *prp20* mutation blocks transport of polyadenylated RNA from nucleus to cytoplasm. In all of these biochemical phenotypes, *prp20* and *rna1* mutants are very similar, if not identical. We discuss several possible interpretations of these data in light of the presumed role of *PRP20* in mitotic regulation, including the possibility that the collection of transcriptional and post-transcriptional phenotypes reflect a defect in the coupling of chromatin structure to mRNA processing.

Results

Inactivation of prp20 causes a decrease in the level of specific mRNAs, and an accumulation of higher molecular weight transcripts

To analyze mRNA production in *prp20* mutants, we prepared total RNA from cells that had been grown continuously at 23°C, or had been shifted from 23°C to 37°C for 30 min (strains are described in Materials and methods and in Table 1). RNAs were separated by agarose-formaldehyde gel electrophoresis, transferred to nylon filters, and hybridized to gene-specific probes. For comparison, we analyzed wild-type and *rna1* strains in parallel. Below, we discuss our results with three mRNAs—*CUP1*, *CYH2*, and *GAL10*.

***CUP1* RNAs** *CUP1* mRNA, which encodes a yeast copper metallothionein, is 520 nucleotides long and does not contain introns (Butt et al. 1984; Karin et al. 1984). Two differences are conspicuous between *CUP1* RNAs from shifted and unshifted *prp20* cultures (Fig. 1A, lanes 3,4). First, the amount of mature *CUP1* mRNA decreases after the shift. Second, much longer transcripts (indicated with arrows in Fig. 1A) accumulate after the shift. These transcripts are ~1–10 kb longer than mature *CUP1* mRNA. Although the decrease in the amount of mRNA could be caused by either a decrease in synthesis or an increase in turnover, the concomitant accumulation of much longer transcripts strongly suggests that mRNA formation is defective. RNAs from an *rna1* mutant strain are very similar to those from *prp20* (lanes 1,2).

We believe that both phenotypes—loss of mature-sized mRNA and accumulation of much longer species—are the result of the mutation in *PRP20*, for two reasons. First, neither phenotype is observed in a wild-type strain (lanes 5,6). Second, we have analyzed five complete tetrads derived from a cross between a *prp20* strain and a wild-type strain by Northern blotting and observe that temperature sensitivity and both Northern blot phenotypes cosegregate in all cases (data not shown).

***CYH2* RNAs** Mature *CYH2* mRNA, which encodes ribosomal protein L29, is 620 nucleotides long and contains a single intron (Kaufer et al. 1983). *CYH2* RNAs detected after a shift of the *prp20* strain exhibit the same defects as seen with *CUP1* (Fig. 1B, lanes 3,4). Specifically, the amount of mature *CYH2* mRNA decreases after the shift and much longer transcripts accumulate (in-

Table 1. *S. cerevisiae* strains

Strain	Genotype	Reference
A364A	<i>MATa ade1 ade2 ura1 tyr1 his7 lys2 gal1</i>	Hartwell (1967)
R14	<i>MATa leu2-3</i>	Winey and Culbertson (1988)
R15	<i>MATa leu2-3</i>	Winey and Culbertson (1988)
M14	<i>MATa ura3-52</i>	M. Culbertson
F9-1d	<i>MATa prp20-1 ura3-52</i>	Fleischmann et al. (1991)
F63-7d	<i>MATa prp20-1 ura3-52</i>	this work
I364	<i>MATa rna1-1</i>	M. Culbertson
F61-6b	<i>MATa rna1-1 ura3-52</i>	this work
F64-3b	<i>MATa rna1-1 ura3-52</i>	this work
Y260	<i>MATa rpb1-1 ura3-52</i>	Nonet et al. (1987)
Y262	<i>MATa rpb1-1 his4-539 ura3-52</i>	Nonet et al. (1987)
F66-10d	<i>MATa rna1-1 rpb1-1</i>	this work
F67-2b	<i>MATa prp20-1 rpb1-1</i>	this work
RL92	<i>MATa prp2 leu2-3 leu2-112 ura3-52</i>	Vijayraghavan et al. (1989)
RL144	<i>MATa prp4 leu2-3 leu2-112 lys2 tyr1 ura3-52</i>	Vijayraghavan et al. (1989)
RL173	<i>MATa prp5 ade his7 leu2 lys2</i>	Vijayraghavan et al. (1989)

licated with arrows). *CYH2* RNAs from the *rna1* mutant yield a very similar pattern of higher molecular weight RNAs (lanes 5,6). Our preliminary results suggest that these extended *CYH2* RNAs are not retained on oligo(dT)-cellulose and so presumably lack poly(A), in contrast to extended transcripts produced from several other genes (St. John and Davis 1981; Aebi et al. 1990). The amount of mature *CYH2* mRNA is lower in *prp20* than in *rna1* (cf. lane 4 with lane 6). After a temperature shift of either mutant, the mature mRNA that remains is slightly shorter. A longer transcript seen in unshifted cultures disappears. In addition, the amount of unspliced precursor decreases dramatically. This may reflect a decrease in the production of *CYH2* RNAs or accelerated degradation of both the precursor and the mRNA.

The phenotypes of *prp20* and *rna1* do not merely result from a splicing defect because they are not observed in three mutant strains that are defective in pre-mRNA splicing, *prp2*, *prp4*, and *prp5* (Fig. 1B, lanes 7–12). RNA prepared from wild-type cultures also changes very little, suggesting that the lesions in *prp20* and *rna1* are responsible for the processing defects (lanes 1,2). This conclusion is confirmed by meiotic segregation of the phenotypes: In five complete tetrads derived from a cross of *prp20* with a wild-type strain, the accumulation of much longer RNAs and the production of slightly shorter, mature-sized mRNA cosegregate, yielding two defective and two wild-type spores in all cases (Fig. 1C). Identical results have been obtained with the *rna1* mutant (not shown).

GAL10 RNAs *GAL10* mRNA, which encodes an enzyme required for utilization of galactose, is 2250 nucleotides long and contains no introns (St. John and Davis 1981). As with *CUP1* and *CYH2*, the amount of mature mRNA decreases after a shift and a longer transcript appears (Fig. 1D, arrow). *prp20* and *rna1* affect the pattern of *GAL10* RNAs similarly.

Summary We conclude that *prp20* and *rna1* display

very similar biochemical phenotypes as assessed by Northern blotting. Defects are observed with three different mRNAs, suggesting that these abnormalities may be of general significance. Decreases in length and amount of mature mRNA and pre-mRNA and even the detailed patterns of much longer transcripts are very similar, if not identical, in *prp20* and *rna1* mutants. These results suggest that the two mutations may affect the same process.

Both transcription initiation and 3'-end formation are defective

We determined whether the longer *CUP1* transcripts seen in the mutants were extended at the 5' or 3' ends. The 5' ends of *CUP1* RNAs were mapped by primer extension (diagram in Fig. 2A). The resulting extension products were analyzed by gel electrophoresis. A prominent product of 240 nucleotides and two less abundant products of 249 and 440 nucleotides were detected in wild-type and unshifted mutant cells (Fig. 2A, lanes 1,2,3,5). The 240- and 249-nucleotide products are consistent with previously mapped 5' termini (Butt et al. 1984; Karin et al. 1984). New products of 280 and 450 nucleotides were produced in *prp20* cells shifted to 37°C (lanes 5,6), indicating that *CUP1* transcripts have altered 5' termini. The same defect was observed in *rna1* cells (lanes 3, 4).

The 3' ends of *CUP1* transcripts were mapped by incubating RNA isolated from mutant cells with a radio-labeled RNA complementary to a region spanning the polyadenylation site of wild-type *CUP1* message. The RNA was then treated with single-strand-specific ribonucleases, and the protected fragments (see diagram in Fig. 2B) were analyzed by gel electrophoresis. RNAs between 105 and 120 nucleotides long were protected by RNA from wild-type and unshifted mutant cells (Fig. 2B, lanes 1,2,3,5). Thus, the 3' termini of *CUP1* mRNAs lie primarily between 189 and 204 nucleotides downstream

Figure 1. Inactivation of *prp20* or *rna1* causes a decrease in the level of specific mRNAs, and an accumulation of higher molecular weight transcripts. RNA was extracted from mutant or wild-type cultures that had been grown continuously at 25°C (odd-numbered lanes) or had been shifted to 37°C for 30 min (even-numbered lanes), as indicated above each lane. RNA was analyzed by Northern blotting using hybridization probes specific for *CUP1* (A), *CYH2* (B,C) or *GAL10* (D). The positions of novel high-molecular-weight species are indicated with arrows and designated 3' extended, in light of later observations. (A) *CUP1* transcripts. (Lanes 1,2) RNA from *rna1* (F64-3b) cells; (lanes 3,4) RNA from *prp20* (F63-7d) cells; (lanes 5,6) RNA from wild-type (R14) cells. (B) *CYH2* transcripts. (Lanes 1,2) RNA from wild-type (A364A) cells; (lanes 3,4) RNA from *prp20* (F9-1d) cells; (lanes 5,6) RNA from *rna1* (1364) cells; (lanes 7,8) RNA from *prp2* (RL92) cells; (lanes 9,10) RNA from *prp4* (RL144) cells; (lanes 11,12) RNA from *prp5* cells. (C) *CYH2* transcripts: meiotic cosegregation of the various RNA-related phenotypes with one another and with temperature sensitivity. *prp20* (F9-1d) and wild-type (R15) cells were crossed and sporulated. The four spores of five different tetrads were dissected and grown at 25°C. Cultures were shifted to 37°C for 30 min. RNA was then prepared and analyzed by Northern blotting using a *CYH2*-specific probe. In each tetrad, two spores were temperature sensitive for growth, whereas two were not, as indicated above each lane. (D) *GAL10* transcripts. (Lanes 1,2) RNA from *rna1* (F64-3b) cells; (lanes 3,4) RNA from *prp20* (F63-7d) cells.

cells; (lanes 3,4) RNA from *prp20* (F63-7d) cells; (lanes 5,6) RNA from wild-type (R14) cells. (B) *CYH2* transcripts. (Lanes 1,2) RNA from wild-type (A364A) cells; (lanes 3,4) RNA from *prp20* (F9-1d) cells; (lanes 5,6) RNA from *rna1* (1364) cells; (lanes 7,8) RNA from *prp2* (RL92) cells; (lanes 9,10) RNA from *prp4* (RL144) cells; (lanes 11,12) RNA from *prp5* cells. (C) *CYH2* transcripts: meiotic cosegregation of the various RNA-related phenotypes with one another and with temperature sensitivity. *prp20* (F9-1d) and wild-type (R15) cells were crossed and sporulated. The four spores of five different tetrads were dissected and grown at 25°C. Cultures were shifted to 37°C for 30 min. RNA was then prepared and analyzed by Northern blotting using a *CYH2*-specific probe. In each tetrad, two spores were temperature sensitive for growth, whereas two were not, as indicated above each lane. (D) *GAL10* transcripts. (Lanes 1,2) RNA from *rna1* (F64-3b) cells; (lanes 3,4) RNA from *prp20* (F63-7d) cells.

of the termination codon. RNA from *prp20* after the shift protected the entire region of homology, which resulted in a band of 207 nucleotides (lane 6). The same pheno-

type was observed in *rna1* cells (lane 4). Therefore, the RNAs produced by the mutants after the shift extend past the normal poly(A) site by at least 100 nucleotides.

Figure 2. Both transcription initiation and 3'-end formation are defective. (A) 5' Termini of *CUP1* transcripts. 5' Termini were determined by primer extension, using a 5'-end-labeled primer that anneals to the position indicated by an asterisk (*). The primer was hybridized to total RNA and extended using reverse transcriptase. The lengths and positions of the major primer extension products are shown. The box indicates the 5' boundary of the *CUP1* open reading frame. Cultures were grown continuously at 25°C (odd-numbered lanes) or shifted to 37°C for 30 min (even-numbered lanes), as indicated above each lane. Numbers to the left of the autoradiograph indicate the positions of molecular weight markers (*MspI*-cleaved pBR322 fragments). (Lanes 1,2) RNA from wild-type (R14) cells; (lanes 3,4) RNA from *rna1* (F64-3b) cells; (lanes 5,6) RNA from *prp20* (F63-7d) cells. (B) 3' Termini of *CUP1* RNAs. 3' Termini were determined by RNase protection, using a uniformly labeled RNA complementary to the region spanning the polyadenylation site of *CUP1* RNAs in wild-type cells (positions +325 to +532). The diagrams illustrate the RNase-resistant products formed with wild-type mRNA (top; lengths of ~120 nucleotides) and with transcripts that extend past the normal polyadenylation site (bottom; ~200 nucleotides). ~ indicates nonhomologous sequences in the polylinker region of the probe. The box indicates the 3' boundary of the *CUP1* ORF. Cultures were grown continuously at 25°C (odd-numbered lanes) or shifted to 37°C for 30 min (even-numbered lanes), as indicated above each lane. Numbers to the left of the autoradiograph indicate the positions of molecular weight markers (*MspI*-cleaved pBR322 fragments). (Lanes 1,2) RNA from wild-type (R14) cells; (lanes 3,4) RNA from *rna1* (F64-3b) cells; (lanes 5,6) RNA from *prp20* (F63-7d) cells.

Presumably, they extend ≥ 1 kb downstream of the normal 3' end, given the lengths observed by Northern blotting (Fig. 1).

To determine whether the 5' and 3' termini of *CYH2* mRNAs are altered in *prp20* and *rna1* strains, we performed RNase protection experiments analogous to those in Figure 2 (data not shown). After a temperature shift of *prp20* and *rna1* strains, *CYH2* 5' termini were detected more than 160 nucleotides upstream of the normal cap site, whereas 3' termini were detected >190 nucleotides downstream of the normal polyadenylation site. These novel 5' and 3' ends were not observed in wild-type strains.

To summarize, transcripts produced after shifting either *prp20* or *rna1* mutants to the restrictive temperature display altered 5' and 3' termini. We conclude that mutations in either of these two genes disrupt both transcription initiation and mRNA 3'-end formation.

Extended transcripts are produced very soon after a temperature shift

To gain insight into whether *prp20* affects 3'-end formation directly, we determined how soon after a temperature shift 3'-end formation went awry. Cells were grown at 23°C and shifted for 0, 1.3, 2.5, 7, or 10 min to 37°C.

Cells were poured over crushed ice and collected by centrifugation, and RNA was isolated immediately. Even after only 1.3 min, extended *CUP1* RNAs were detected in *prp20* (Fig. 3, lane 7). Similar amounts of longer transcripts were detected in *rna1* even at the shortest time points. The amount of extended transcript increased until 2.5 to 7 min, when it reached a maximum.

The very rapid appearance of long transcripts after a shift argues strongly against models in which the observed effects require synthesis of a new factor. It is consistent with a cotranscriptional defect in mRNA 3'-end formation.

Shortening of mature-sized RNAs is due to loss of poly(A) from pre-existing mRNAs

Mature-sized *CYH2* RNA is 20–30 nucleotides shorter in *prp20* and *rna1* cells following a shift to the restrictive temperature (Fig. 1B, lanes 4,6). A similar decrease in length is seen with *CYC1* and ribosomal protein L32, L4, and S7 mRNAs (not shown). To test whether the decrease in length was due to loss of poly(A), total RNA from wild-type, *prp20*, and *rna1* cells was incubated with an anti-*CYH2* oligonucleotide and RNase H, with or without oligo(dT) (diagram in Fig. 4). RNase H cleaves the *CYH2* message to produce a 198-nucleotide 3' fragment as a result of the annealing of the anti-*CYH2* oligonucleotide and removes poly(A) only in the presence of oligo(dT). The size of the resulting 3' fragments of the mRNA were determined by Northern blotting.

The poly(A) tail of *CYH2* mRNAs in wild-type, *prp20*, and *rna1* cells, grown at 23°C is ~20–30 nucleotides (Fig. 4, lanes 1,2,5,6,9,10). The *CYH2* poly(A) tail is very short or missing after a shift of *prp20* and *rna1* cells (Fig. 4, lanes 7,8,11,12). The amount and length of mature *CYH2* mRNA decreases progressively in both *prp20* and *rna1* following a shift to the restrictive temperature (data not shown).

The lack of poly(A) on *CYH2* mRNA after a temperature shift could be the result of deadenylation of mRNA that was present before the shift or de novo synthesis of

poly(A)-deficient RNAs after the shift. To distinguish between these possibilities, we determined whether shortening was observed when transcription was blocked during the temperature shift. To block transcription, we generated double mutants containing *rpb1*, a temperature-sensitive mutation in a subunit of RNA polymerase II (Nonet et al. 1987), with either *prp20* or *rna1*. In an *rpb1* mutant, *CYH2* mRNA decreases in abundance and length as expected from its reported half-life and deadenylation (Fig. 5, lanes 3,4; Herrick et al. 1990). The amount and length of *CYH2* mRNA decreases in *prp20 rpb1* and *rna1 rpb1* double mutants (lanes 7–10). The amount of mRNA remaining differs between strains, most likely reflecting effects on mRNA turnover in the different backgrounds. Accumulation of the longer transcripts is also blocked in the double mutants, demonstrating that those RNAs result from transcription only after the shift.

We conclude that the poly(A)[−] *CYH2* mRNA observed after the temperature shift was produced by deadenylation of pre-existing mRNA. We presume that this reflects normal *CYH2* mRNA metabolism but is particularly visible in the mutants because the formation of new, mature-sized mRNA is blocked.

Bulk poly(A) length distribution changes after a temperature shift

rna1 mutants accumulate mRNA with longer poly(A) tails after a shift to the restrictive temperature (Piper and Aamand 1989). It has been suggested that this reflects a defect in transport of mRNA from nucleus to cytoplasm, such that nuclear mRNAs, with long poly(A) tails, are not exposed to the cytoplasmic poly(A) removal apparatus (Hutchison et al. 1969; Shiokawa and Pogo 1974; Piper and Aamand 1989).

To determine whether *prp20* might also impair mRNA transport, we first measured poly(A) length in total RNA extracted from *prp20* cells after a shift. Total RNA was treated with ribonuclease to degrade sequences other

Figure 3. Extended transcripts are produced very soon after a temperature shift. RNA was prepared from *rna1* (F64-3b) or *prp20* (F63-7d) cells that had been shifted to 37°C for varying lengths of time, as indicated above each lane. *CUP1*-specific transcripts were detected by Northern blotting using a *CUP1*-specific probe. (Lanes 1–5) RNA from *rna1* cells; (lanes 6–10) RNA from *prp20* cells.

Figure 4. Shorter *CYH2* RNAs after a temperature shift lack poly(A). The length of poly(A) present on *CYH2* RNAs was determined using the oligonucleotide/RNase H protocol diagrammed at top. RNAs were incubated with RNase H and an oligonucleotide complementary to nucleotides +837 to +861 of *CYH2* mRNA, thereby generating short (~200 nucleotides) 3' fragments of *CYH2* mRNAs. Poly(A) tails were removed from half of each sample, using oligo(dT) and RNase H. RNAs were analyzed by Northern blotting, using a *CYH2*-specific probe extending from +840 to +1300. The length of poly(A) is calculated from the difference in length between samples incubated with or without oligo(dT), as indicated above each lane. The duration of the growth at 37°C is indicated above each lane as well. Ten micrograms of total RNA was used per lane, except in lanes 3, 4, 11, and 12, in which 20 µg of RNA was used to normalize signal. (Lanes 1–4) RNA from wild-type (R14) cells; (lanes 5–8) RNA from *rna1* (F64-3b) cells; (lanes 9–12) RNA from *prp20* (F63-7d) cells.

than poly(A). The remaining RNA fragments were radiolabeled and analyzed by gel electrophoresis.

The distributions of poly(A) lengths in wild-type cells at 23°C or 37°C are very similar (Fig. 6, lanes 1,2). The poly(A) lengths present in RNA from *prp20* and *rna1* cells grown at 23°C are comparable to wild type (lanes 3,5). After a shift, however, both *prp20* and *rna1* strains accumulate RNA with longer poly(A) tails, with peak lengths of between 60 and 90 nucleotides (lanes 4,6). The peak length is slightly shorter with *prp20* than with *rna1*. Concomitantly, poly(A) tails of ≤60 nucleotides decrease in abundance. The loss of shorter poly(A) tails is consistent with the deadenylation of *CYH2* mRNA seen after a shift to the restrictive temperature (see Fig. 4).

These results suggest that *prp20* cells may accumulate

newly synthesized mRNA in the nucleus. The increase in longer poly(A) tails could reflect newly synthesized poly(A) that is protected from cytoplasmic deadenylation by retention in the nucleus, whereas the decrease in shorter poly(A) tails could result from the deadenylation of mRNAs that were already in the cytoplasm before the temperature shift.

Poly(A) accumulates in the nucleus in prp20 and rna1 cells after a temperature shift

To test directly whether poly(A) accumulates in the nucleus after a temperature shift of *prp20* and *rna1* cells, we analyzed the distribution of bulk poly(A) by in situ hybridization. Cells were permeabilized, fixed, and hybridized to digoxigenin-labeled oligo(dT). The oligo(dT) was visualized using rhodamine-conjugated anti-digoxigenin antibody. The location of the nucleus in each cell was determined by DAPI staining.

The nuclei of *prp20* or *rna1* cells shifted to 37°C for 30 min stain prominently with oligo(dT) (Fig. 7). In unshifted cultures, the staining is diffuse and cytoplasmic; nuclei are conspicuously unstained (Fig. 7). Cytoplasmic staining decreases after the shift. The nuclear staining observed at 37°C is somewhat less intense with *prp20* than with *rna1* cells. Approximately 50% of *prp20* cells show increased nuclear staining, as compared with nearly 100% with *rna1*.

From these data, we conclude that *prp20* and *rna1* result in the accumulation of poly(A) in the nucleus. We infer that like *rna1*, *prp20* mutants are defective in mRNA transport to the cytoplasm.

Discussion

The results presented in this report indicate that a temperature-sensitive lesion in the *PRP20* gene results in several defects in mRNA metabolism. First, mature mRNA levels decrease after a shift to the restrictive temperature. In addition to the three mRNAs analyzed here (Fig. 1), seven other genes yield similar results (data not shown), suggesting that this phenotype is common to many genes. Second, the formation of 3' termini is defective: 3' termini are formed at sites 1–10 kb downstream of the wild-type polyadenylation site of three different mRNAs. This result, reported previously for a single RNA (Aebi et al. 1990), has been extended here to include several other transcripts and has been confirmed by RNase mapping experiments. Longer transcripts also are observed with the *TCM1* and *PAB* genes (Aebi et al. 1990), but these have not been characterized biochemically. Thus, the defect in 3'-end formation is likely to occur on many genes in *S. cerevisiae*. Third, transcription initiation is perturbed because *CUP1* and *CYH2* transcripts possess novel 5' termini. Fourth, transport of mRNA from the nucleus to the cytoplasm is defective, as indicated by in situ hybridization to oligo(dT). Fifth, the distribution of total poly(A) tail lengths changes after a temperature shift, with a dramatic increase in the abundance of longer lengths and a decrease in the abun-

Figure 5. *CYH2* mRNA without poly(A) is derived from RNA produced prior to the temperature shift. RNAs were analyzed by Northern blotting, using a hybridization probe complementary to the *CYH2* gene (see Materials and methods). RNA was prepared from cells that had been grown continuously at 25°C (odd-numbered lanes) or that had been shifted to 37°C for 30 min (even-numbered lanes), as indicated above each lane. (Lanes 1,2) RNA from wild-type (R14) cells; (lanes 3,4) RNA from *rpb1* (Y260) cells; (lanes 5,6) RNA from *prp20* (F63-7d) cells; (lanes 7,8) RNA from *rna1* (F61-6b) cells; (lanes 9,10) RNA from the *rpb1 rna1* double mutant (F66-10d); (lanes 11,12) RNA from the *rpb1 prp20* double mutant (F67-2b). Extended transcripts are indicated by arrows. The material detected in wild-type cells near the positions of the 3'-extended transcripts in rRNA, as indicated to the right of the autoradiograph.

dance of shorter lengths. In addition to these phenotypes, *prp20* also is defective in the splicing of actin pre-mRNA (Vijayraghavan et al. 1989). Splicing of *CYH2* mRNA is not obviously impaired (Fig. 1B), but this could reflect accelerated degradation of the unspliced pre-mRNA, as is observed with mature *CYH2* mRNA. Finally, the *prp20* mutation may also impair transcription termination because it has been reported that termination in yeast occurs quite close to poly(A) addition sites (Osborne and Guarente 1989).

Some of these defects are likely to be the result of a lesion at an upstream step in mRNA synthesis or maturation. For example, a failure to transport mRNA would account for the increase in longer poly(A) lengths. The transport defect itself could be the result of a failure in 3'-end formation. Similarly, the decrease in the amount of mature mRNA probably reflects the defects in initiation and 3'-end formation: Because mature-sized mRNA is no longer efficiently formed after the temperature shift, the decay of mRNA that was present before the shift becomes visible. This would account for the decrease in the average length of poly(A) on specific mRNAs, as well as the decreased abundance of short, cytoplasmic poly(A).

Quantitative differences between transcripts are likely to be the result of differing rates of synthesis and degradation, characteristic of those RNAs. mRNAs differ in decay rates (Herrick et al. 1990), which could explain the variable rates of decay observed after a shift. Alternatively, the supply of mature-sized mRNAs could be more severely impaired with one transcript than another. Although three genes produce novel, long transcripts extended at their 3' ends, the quantity of these extended transcripts varies considerably between genes. Again, this presumably reflects differences in the intrinsic stability of these aberrant RNAs and in the rate with which each gene is transcribed to produce the aberrant RNA.

We have assayed *rna1* and *prp20* mutant phenotypes in parallel and observe that the two mutations behave very similarly. Particularly striking are qualitative aspects of

the data. For example, the distribution of specific initiation sites and of extended transcripts is very similar, if not identical, in the two mutants. Thus, the differences between genes are likely to be gene-specific responses to the same defect and not a consequence of either *prp20* or *rna1*. In some experiments, low levels of aberrant initiation and termination events are detectable even in the wild-type strain, suggesting that *prp20* and *rna1* enhance an intrinsic gene-specific property. *prp20* and *rna1* could either increase the frequency of the rare event, or decrease the frequency of the common, wild-type event. In the particular case of 3' end formation, the mutants could decrease the frequency of proper 3'-end cleavage from near 100% to some lower level. This change would cause a dramatic increase in the abundance of transcripts with 3' extensions and would yield a pattern of extended transcripts that varies from one gene to another.

The *PRP20* protein is nuclear (Fleischmann et al. 1991), whereas the *RNA1* protein is cytoplasmic (Hopper et al. 1990). Although the predominantly cytoplasmic location of *RNA1* protein complicates explanations of how the *rna1* allele disrupts nuclear events, a fraction of the protein could be nuclear, or *RNA1* protein could be involved in some form of cytoplasmic-nuclear signaling, as proposed by Hopper et al. (1990). Although the great similarity in the phenotypes of *rna1* and *prp20* suggests that they might affect the same process, we observe no synthetic lethality in *rna1 prp20* double mutants (not shown).

The similarities between *PRP20* and the human gene, *RCC1*, and an *S. pombe* gene, *PIM1*, suggest that all of the RNA-related phenotypes of *prp20* mutants may be consequences of improper regulation of the cell cycle and/or chromosome structure. *PRP20* is 30% identical over a 474-amino-acid stretch with human *RCC1* protein and *S. pombe* *PIM1* protein (Aebi et al. 1990; Matsumoto and Beach 1991). Each member of this family contains 7 repeats of a 60-amino-acid domain (Ohtsubo et al. 1987). Mammalian cells that are homozygous for a point mutation in *RCC1* undergo premature chromo-

Figure 6. Bulk poly(A) length distribution changes after a temperature shift. Poly(A) length distributions were determined by digesting total RNAs to completion with RNases T1 and A. The resistant oligo(A) and poly(A) segments were then 3'-end-labeled using RNA ligase and [32 P]pCp. RNA was prepared from cells that had been grown continuously at 25°C (odd-numbered lanes) or had been shifted to 37°C for 30 min (even-numbered lanes), as indicated above each lane. The positions of DNA length markers (*Msp*I fragments of pBR322) are indicated to the left of the autoradiograph. (Lanes 1,2) RNA from wild-type (R14) cells; (lanes 3,4) RNA from *rna1* (F64-3b) cells; (lanes 5,6) RNA from *prp20* (F63-7d) cells.

some condensation at the restrictive temperature (Uchida et al. 1990). Similarly, the *S. pombe* *pim1* mutation was identified as a temperature-sensitive lethal lesion that caused premature chromosome condensation and mitotic spindle formation after a shift to the restrictive temperature (Matsumoto and Beach 1991). *S. cerevisiae* strains that lack *PRP20* also display alterations in

both the nucleolus and the nucleoplasm after a temperature shift, demonstrating that it too results in a perturbation of nuclear or chromosomal structure (Clark and Sprague 1989; Aebi et al. 1990). Because the mammalian *RCC1* gene can rescue the temperature-sensitive lethality of a *prp20* mutant strain, and the *PRP20* gene can rescue *RCC1*-defective mammalian cells (Clark et al. 1991; Fleischmann et al. 1991; Ohtsubo et al. 1991), these two genes are likely to have overlapping functions.

The defects observed in *rcc1* and *pim1* mutants probably arise from misregulation of entry into mitosis. For example, *pim1* mutants enter mitosis directly from G₁, in a process that requires the *CDC2* gene product (Matsumoto and Beach 1991). A high-copy suppressor of *pim1*, called *SPI1*, itself affects entry into mitosis and chromosome condensation (Matsumoto and Beach 1991). *S. pombe* *SPI1* is very similar in sequence to the mammalian gene *RAN* (Matsumoto and Beach 1991), with which *RCC1* protein appears to interact biochemically (Bischoff and Ponstigl 1991). Both *SPI1* and *RAN* are nuclear, *ras*-like proteins; their homolog in *S. cerevisiae*, if any, has not yet been identified.

On the basis of the putative function of the *PRP20*–*PIM1*–*RCC1* gene family, we suggest two distinct models for the role of *PRP20* in mRNA maturation. In the first model, *PRP20* participates directly in several different RNA-processing steps. The global disruptions of mRNA maturation in the mutants happen to manifest themselves as cell cycle defects. For example, in *S. pombe*, because *CDC2* is required for the *pim1* phenotype (Matsumoto and Beach 1991), changes in the metabolism of this or another functionally related mRNA could cause premature mitosis. Consistent with this view, the RNA-processing defects of *prp20* in *S. cerevisiae* are detected after only a few minutes (Fig. 3), whereas mitotic defects are observed only after hours with *pim1* in *S. pombe* (Matsumoto and Beach 1991). We find this hypothesis unattractive, however, both because it requires that a single gene product participate directly in a wide diversity of RNA processing steps and that global disturbances in mRNA metabolism result in a very similar phenotype (entry into mitosis) in distantly related organisms.

A second hypothesis proposes that all of the RNA-related phenotypes of the *prp20* mutation are secondary consequences of a primary defect in a single, upstream target. Chromatin—in particular, the structure of the chromatin–nascent ribonucleoprotein (RNP)–transcription complex—is a particularly attractive possibility for such a target, in light of the chromosome condensation effects described above. Our observation that sites of transcription initiation are altered in *prp20* mutants is readily explained by a change in chromatin structure. This hypothesis also is consistent with the very rapid appearance of aberrant 3' termini after a shift to 37°C (Fig. 3) and the notion that mRNA 3'-end cleavage occurs cotranscriptionally (Nevins and Darnell 1978; Logan et al. 1987; Connelly and Manley 1988). In this context, the splicing defect in *prp20* mutants (Vijayraghavan et al. 1989) would imply that some aspect of spliceosome as-

Figure 7. Poly(A) accumulates in the nucleus of *prp20* and *rna1* cells after a temperature shift. *rna1*(F64-3b) or *prp20* (F63-7d) cells were grown continuously at 25°C (first and third columns) or were shifted to 37°C for 30 min (second and fourth columns), before fixing and staining. The three photos in each column show the same field of cells. The *top* row shows the cells viewed by phase-contrast microscopy (bar, 10 μ m). The *middle* row shows the same cells stained with oligo(dT)-digoxigenin and probed with anti-digoxigenin antibody conjugated with rhodamine and viewed by fluorescence. The *bottom* row shows the cells stained with DAPI and viewed by fluorescence to identify the position of the nucleus.

sembly occurs cotranscriptionally, a notion for which there is evidence in both *Drosophila* (Beyer and Osheim 1988; LeMaire and Thummel 1990) and in yeast (D.J. Elliott and M. Rosbash, unpubl.). The effect of *prp20* on mRNA transport from nucleus to cytoplasm might be an independent manifestation of the chromatin defect (e.g., improper cotranscriptional assembly of an RNP complex required for transport) or could be a consequence of improper 3'-end formation (Eckner et al. 1991). 3'-End formation is not detectably perturbed *in vivo* in extracts derived from *prp20* mutant cells (S. Butler, pers. comm.), suggesting that *PRP20* may act upstream of mRNA processing *per se*. An alternative primary defect in *prp20* mutants could lie in regulation of the cell cycle. Again, this primary defect could lead to secondary defects in many different RNA-processing steps. *cdc2* is epistatic to *pim1* (Matsumoto and Beach 1991), and *RCC1* mutants can affect *CDC2* kinase activity (Nishitani et al. 1991). Thus, the mRNA maturation defects seen in *prp20* mutants could be caused by inappropriate regulation of *CDC28* or another regulator of the cell cycle. For example, loss of *prp20* function could activate the *CDC28* kinase. In turn, this misregulated activity could then modify multiple targets involved in mRNA 3'-end formation, transcription initiation, splicing, and transport.

These targets for modification might be recognized in the normal transition from G_2 to M, or they could be modified only in the aberrant situation in which cells actively transcribing and processing RNAs enter mitosis directly from G_1 or S. Finally, we note that primary defects in processes other than chromatin structure or entry into mitosis, on which we have focused here, are also consistent with the data.

Our results raise the possibility that mRNA processing and chromatin structure or mitotic control are coupled but do not establish the nature of the connection. To elucidate the primary function of *PRP20*, it will be necessary to identify the components with which it interacts *in vivo*. These components could participate directly in mitotic control, chromatin condensation, or mRNA processing and may include homologs of *S. pombe SPI1* and human *RAN*. It will be necessary to establish an *in vitro* system in which mRNA processing is sensitive to *PRP20* protein. To this end, cell-free systems in which mRNA processing is coupled to transcription of chromatin may be essential. Finally, an examination of mRNA processing in *cdc28* mutants, and of mitotic regulation in *rna1* mutants, may help illuminate whether the defects in mRNA processing are primary or secondary consequences of improper cell cycle control.

Materials and methods

Yeast strains

Strains are listed in Table 1. Lowercase italics (e.g., *prp20*) are used to designate mutant genes; uppercase italics (e.g., *PRP20*) indicate wild-type genes. For clarity, we have used this same convention regardless of species. Strain 1364 was crossed sequentially with strain M14 and R15 to generate F61-6b and F64-3b. F9-1d was crossed with R15 to generate F63-7d. F66-10d was generated by crossing F62-9c with Y260. F67-2b was generated by crossing F63-7d with Y262. The spores analyzed (Fig. 1C and data not shown) were from crosses of *prp20* (F9-1d) with wild-type (R15) (five complete tetrads) and of *rna1* (F61-6b) with wild-type (R14) (eight complete tetrads). Cultures were grown and RNA was analyzed as described below. All crosses were performed using standard yeast techniques. Cultures were grown in YPD medium or in SC with 2% galactose in place of dextrose (Sherman 1991) to induce expression of the galactose utilization genes.

Plasmids

Plasmid pWF1 was constructed by cloning an *XbaI*–*KpnI* genomic restriction fragment extending from position –228 to +475 (numbers are relative to the A of the AUG) of the *CUP1* gene into pGEM3Z (Promega). Plasmid pWF6 contains a *HincII*–*EcoRI* fragment extending from position –400 to +860 of the *CYH2* gene inserted into pGEM3Z. Plasmid pWF38 was generated by ligating an *EcoRI*–*SalI* fragment encompassing much of the *GAL10* open reading frame [from plasmid pNN76 (St. John and Davis 1981), provided by M. Johnston] into pGEM3Z. Plasmid pWF36 was constructed by ligating a *BglII*–*PstI* fragment from +840 to +1300 of the *CYH2* gene (from plasmid YEp-CYH-U1, provided by H. Fried, University of North Carolina, Chapel Hill) into pGEM3Z. Plasmids were grown in *Escherichia coli* strain JM109, and DNA was extracted as described (Sambrook et al. 1989). Restriction digests were performed as recommended by the suppliers (Promega, New England Biolabs).

RNA isolation, Northern blot analysis, and RNase H ablation

Cultures (generally 5 or 15 ml) were grown to an OD₆₀₀ of 1 in YPD at room temperature and were shifted to a 37°C water bath for the indicated length of time, then cooled in ice water prior to RNA extraction. For cultures shifted 10 min or less, 5-ml cultures were grown to an OD₆₀₀ of 1 and quickly mixed with an equal volume of YPD prewarmed to 51°C and incubated at 37°C. Cultures were poured over crushed ice. Cells were harvested by centrifugation and RNA extracted using the glass bead lysis method (Ausubel et al. 1987).

For Northern blot analysis, total RNA (10 µg) was fractionated on 2% agarose–formaldehyde gels (Sambrook et al. 1989) and transferred to ZetaProbe nylon membrane (Bio-Rad) with 10× SSC. Gene-specific RNA probes were synthesized using T7 (pWF1 and pWF6) and SP6 (pWF36 and pWF38) viral RNA polymerases (New England Biolabs, Promega) according to the suppliers' protocols. Prehybridization was in 50% formamide (vol/vol), 1× SSPE, 1% SDS, 0.5% Carnation nonfat dry milk, 0.5 µg/ml of denatured salmon sperm DNA at 65°C as recommended by the supplier (Bio-Rad). Dextran sulfate to 5% (wt/vol) and 10⁶ cpm/ml of RNA probe were added for 12–16 hr at 65°C. Filters were washed twice in 2× SSC, 0.1% SDS, and once in 0.2× SSC, 0.1% SDS, at 65°C and exposed to Kodak XAR5 film.

For measurement of poly(A) tail length, oligo anti-CYH2 (5'-TCTACCCTTACCCAAGATCTTACC-3'), complementary to

position +837–+861 was mixed with duplicate RNA samples and oligo(dT)_{12–18} was added to one of each of these samples. RNase H cleavage was carried out as described (Vournakis et al. 1975). The resulting fragments were analyzed by Northern blot as described above except that 4% NuSeive agarose was used. The probe was generated as an SP6 runoff from plasmid pWF36, linearized with *EcoRI*.

Mapping 5' and 3' termini

The 5' ends of CUP1 transcripts were mapped by primer extension. Ten nanograms of 5'-end-labeled anti-CUP1 primer (5'-TCATTTCCCAGAGCAGCATG-3'), complementary to position +166 to +186 was incubated with 5 µg of total RNA from yeast in 7.5 µl at 42°C for 60 min. Fresh extension mix (0.5 mM each deoxyribonucleotide, 40 mM MgCl₂, 4 mM dithiothreitol, 2.5 mg/ml of actinomycin D, and 6 units of AMV reverse transcriptase) was added and tubes were incubated 30 min at 37°C. Reactions were stopped by the addition of 8 µl of sequencing gel loading mix; samples were boiled for 2 min, and half was loaded on a 6% polyacrylamide sequencing gel.

3' Termini were mapped by RNase protection, essentially as described (Sambrook et al. 1989). A 220-nucleotide probe complementary to +325 to +532 was generated by transcription of *DdeI*-cut plasmid pWF1 using T7 RNA polymerase. Total yeast RNA (20 µg) was incubated in 30 µl of 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% formamide with 1.5 × 10⁵ cpm probe for 10 min at 85°C, followed by 16 hr at 48°C. Then 300 µl of RNase mix (300 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA, 0.5 units of RNase T1, 10 ng of RNase A) was added, and samples were incubated for 60 min at 30°C. Twenty microliters of 10% SDS and 10 µl of 10 mg/ml proteinase K was added, and samples were incubated for 30 min at 37°C. Samples were extracted once with 50 : 50 phenol : chloroform and ethanol precipitated. Pellets were dried, resuspended in sequencing gel loading solution and analyzed by electrophoresis in 8% polyacrylamide sequencing gel.

Bulk poly(A) length determination

Total RNA was treated with 2 mg/ml of bovine pancreatic ribonuclease and 1000 U/ml of RNase T1 to hydrolyze all sequences other than poly(A). RNA was labeled by incubation with 100 µCi of 5'-³²P cytidine 3',5'-bis(phosphate) (NEN Dupont) in 50 mM Tris-HCl (pH 7.9), 15 mM MgCl₂, 3.3 mM dithiothreitol, 2% (vol/vol) dimethyl sulfoxide, 10 mg/ml of bovine serum albumin, 25 µM ATP, and 10 units of T4 RNA ligase (New England Biolabs) for 21 hr at 4°C. RNA samples were extracted with phenol/chloroform and ethanol precipitated. Pellets were resuspended in 96% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol, boiled for 2 min, and loaded onto a 10% polyacrylamide/7 M urea gel in TBE. After electrophoresis, the gel was dried and exposed to Kodak XAR5 film.

Staining by in situ hybridization

In situ hybridizations and immunofluorescence stainings were performed as described (Kadowaki et al. 1992), with several modifications. Cultures were grown to an OD₆₀₀ of 0.5–1 and heated for 30 min at 37°C, where required. Cells were fixed with 4% formaldehyde for 45 min, washed three times in solution A [1.2 M sorbitol, 100 mM KPO₄ buffer (pH 7.5)], and spheroplasted for 20 min at 30°C in solution A containing 0.1 mg/ml of oxalolyticase, 30 mM β-mercaptoethanol, 60 µg/ml of PMSF, and the protease inhibitors aprotinin, leupeptin, and pepstatin at 1 µg/ml. Cells were washed in solution A and spotted onto poly-

L-lysine-coated coverslips for 30 min at 4°C, washed again, and dehydrated in 70% ethanol overnight. In situ hybridization was performed overnight at 37°C in 20 µl of a mix containing 10–20 ng of oligo(dT)_{25–30} (Pharmacia), 3'-end-labeled with digoxigenin-11-dUTP (Boehringer Mannheim) and terminal transferase (BRL), 20 µg of salmon sperm DNA, 20 µg of tRNA, 2× SSC, 0.2% BSA, 10 mM vanadyl ribonucleoside complexes, and 10% dextran sulfate. The coverslips were washed three times in 2× SSC at 37°C and three times in 1× SSC at room temperature (10 min per wash); they were treated for 5 min with buffer B [150 mM NaCl, 100 mM Tris (pH 7.5)] containing 0.1% Triton and rinsed twice with buffer B. The coverslips were incubated for 60 min at room temperature with a sheep polyclonal anti-digoxigenin antibody conjugated with rhodamine (Boehringer Mannheim) in buffer B containing 1% BSA. The coverslips were washed 15 min in buffer B and 15 min in 300 mM NaCl, and 100 mM Tris (pH 7.5) and mounted on slides with one drop of 90% glycerol, containing 1 mg/ml of *p*-phenylenediamine, 100 ng/ml of 4', 6-diamido-2-phenylindole (DAPI), and PBS. Pictures were taken with a Zeiss Axiophot microscope.

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