RNA Polymerase III Defects Suppress a Conditional-Lethal Poly(A) Polymerase Mutation in *Saccharomyces cerevisiae*

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ABSTRACT

We isolated spontaneous extragenic suppressors of a temperature-sensitive, lethal poly(A) polymerase mutation (*pap1-1*) in *Saccharomyces cerevisiae* that restore growth at the restrictive temperature of 30°. Three of five suppressors represent alleles of the *PDS2* complementation group. The recessive *pds2-1* mutation exerts dominant allele-specific suppression over *pap1-1*, suggesting a direct functional interaction. The suppressor restores to near normal the steady-state concentrations of various mRNAs and total poly(A) reduced by *pap1-1* at 30°. Transcriptional chase experiments detect no reduction in the decay rates of mRNAs in the suppressor strain, suggesting that the restoration of steady-state message levels results from increased stable mRNA synthesis. Molecular cloning shows *PDS2* to be allelic to *RET1*, which encodes the second-largest subunit of RNA polymerase III. We observe alterations in both the length and the steady-state amounts of RNA polymerase III transcripts in *pds2-1* strains. Previously identified *ret1* alleles do not suppress *pap1-1*, indicating that the *pds2* alleles we isolated represent a specific class of *RET1* mutations that suppress *pap1-1*. Suppression of *pap1-1* by mutations in an RNA polymerase III subunit suggests a number of potentially novel interactions between these enzymes.

EUKARYOTIC messenger RNA must undergo a number of maturation events before exerting its effect on the cell through translation. These modifications include the addition of a 5′ cap structure, splicing of introns, and 3′ end cleavage and polyadenylation of the primary transcript. In higher organisms, site-specific endonucleolytic cleavage and polyadenylation determine the mature mRNA's 3′ end for all transcripts except those of histones, which exhibit a complex processing event distinct from general 3′ end formation (MOWRY *et al.* 1989). In *Saccharomyces cerevisiae,* conversely, all nuclear-encoded mRNAs examined display a poly(A) tail of ~50–70 adenosines. Considering its near ubiquity throughout eukaryotes, the processing event at the 3′ end of pre-mRNA must have an important, if elusive, role in the cell.

A large body of evidence suggests that the physiological roles of poly(A) tails include contributions to nuclear and cytoplasmic mRNA stability, nucleocytoplasmic transport and localization, and enhancement of translational initiation or reinitiation (BRAWERMAN 1981; HERRICK *et al.* 1990; PATEL and BUTLER 1992; DECKER and PARKER 1993; PROWELLER and BUTLER 1994; COHEN 1995; O'HARA *et al.* 1995). We previously characterized a conditional mutation in the gene encoding yeast poly(A) polymerase (*pap1-1*) and shed light on the role of the poly(A) tail in yeast (PROWELLER and BUTLER 1994). Both poly(A)-deficient mRNAs and mRNAs totally lacking poly(A) tails associated with large polyribosomes, indicating that neither cellular localization nor efficient translation of mRNA unconditionally requires a poly(A) tail. Indeed, the rapid loss of mRNA from the steady-state pool of transcripts, presumably due to a decrease in stability caused by the inability to synthesize a poly(A) tail, was the primary physiological defect observed in *pap1-1* cells at 35°. This is supported by recent evidence that nuclease-driven deadenylation comprises the rate-limiting step in the cytoplasmic decay of many normal mRNAs (BEELMAN and PARKER 1992; DECKER and PARKER 1993; HSU and STEVENS 1993; MUHLRAD *et al.* 1994).

The mechanism of mRNA cleavage and polyadenylation is quite complex. Fractionation of processing extracts competent for site-specific cleavage and polyadenylation has led to the identification of at least 10 polypeptides required for accurate 3′ end processing in mammalian cells. In yeast, purification of poly(A) polymerase and identification of a conditional defect in the enzyme each led independently to the cloning of the PAP1 gene (LINGNER *et al.* 1991; PATEL and BUTLER 1992). A variety of genetic and biochemical strategies allowed the characterization of four other factors required for efficient mRNA 3′ end processing, revealing homology between some of the factors in yeast and humans (MINVIELLE-SEBASTIA *et al.* 1994; TAKAGAKI and MANLEY 1994; PREKER *et al.* 1995; RUSSNAK *et al.* 1995; for recent review, see KELLER 1995). The role of this processing complex (as in the mammalian system) is to recognize a specific site on the newly transcribed (or

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TABLE 1

Yeast strains used in this study

Strain	Relevant genotype or description	Source or reference
A364A	*MAT***a**, *ade1, ade2, ura1, his7, lys2, tyr1, gal1*	PATEL and BUTLER (1992)
UR3148-1B	*MATα, ade1 and/or ade2, lys2, gal1, ura3-52, pap1-1*	PATEL and BUTLER (1992)
BPO2	*PAP1* strain isogenic to UR3148-1B	This study
UR3148-6A	*MAT***a**, *leu2-3, ura3-52, pap1-1*	This study
UR3148-1BC1	*MATα, ade1 and/or ade2, lys2, gal1, ura3-52, pap1-1, pds1-1*	This study
UR3148-1BC2	*MATα, ade1 and/or ade2, lys2, gal1, ura3-52, pap1-1, pds2-1*	This study
UR3148-1BC10	*MATα, ade1 and/or ade2, lys2, gal1, ura3-52, pap1-1, pds2-2*	This study
UR3148-1BC11	*MATα, ade1 and/or ade2, lys2, gal1, ura3-52, pap1-1, pds2-3*	This study
UR3148-1BC12	*MATα, ade1 and/or ade2, lys2, gal1, ura3-52, pap1-1, pds1-2*	This study
S288C	*MAT***a**, *gal2*	
ABC2-2D	*MATα, ade1 and/or ade2, lys2, his7, ura3-52, pds2-1*	This study
Wx142-5D	*MAT***a**, *trp1-7, ura3-52, pap1-10*	MARK WINEY
2D142-2A	*pap1-10, PDS2* segregant of ABC2-2D crossed to Wx142-5d	This study
2D142-2D	*pap1-10, pds2-1* segregant of ABC2-2D crossed to Wx142-5d	This study
6AC2	UR3148-6A crossed to UR3148-1BC2	This study
1B6A	UR3148-1B crossed to UR3148-6A	This study
ABC2	A364A crossed to UR3148-1BC2	This study
SA23-1A	*MATα, trp1, ura3, met4, lys2, ade2, leu2, his3, ret1::HIS3, can1, cyh2*, carrying pSA23	NICK ZECHERLE
SAI11	Isogenic to SA23-1A with pSA23 replaced by pBK-I11	This study
SAI15	Isogenic to SA23-1A with pSA23 replaced by pBK-I15	This study
1BTH-3D	*MATa, ade1 and/or ade 2, lys2, trp1-901, his3-200, pap1-1, RET1*	This study
1BTHI11	Isogenic to 1BTH-3D with pBK-I11	This study
1BTHI11 Δret	*HIS3* disruption of chromosomal *RET1* allele of 1BTHI11	This study
1BTHI15	Isogenic to 1BTH-3D with pBK-I15	This study
1BTHI15 Δret	*HIS3* disruption of chromosomal *RET1* allele of 1BTHI15	This study

transcribing) premessage, endonucleolytically cleave the pre-mRNA at that site, and catalyze the addition of adenosines to the free 3′-OH formed (for comprehensive reviews, see MANLEY 1988; WAHLE and KELLER 1992; BIENROTH *et al.* 1993).

The extensive characterization of the mammalian processing complex has relied heavily on the biochemical fractionation of cell free extracts competent for processing. While this has also proved a fruitful strategy in understanding the yeast system, researchers of this organism have the added advantage of a powerful genetic system. We have undertaken a pseudoreversion analysis of the *pap1-1* mutation with the goal of isolating genes whose products are directly involved in the mechanism or function of polyadenylation in yeast. We discovered suppressors that represent a subset of mutations in a core subunit of RNA polymerase III. These mutations partially suppress the *pap1-1*- associated defect in the maturation of mRNAs transcribed by RNA polymerase II and may indicate an unprecedented relationship between RNA polymerase III and the mRNA 3′ end processing machinery.

MATERIALS AND METHODS

Strains, media, and genetic techniques: The experiments reported here were performed using strains described in Table 1. Strain BPO2 is isogenic to UR3148-1B (save for the *PAP1* allele) and was created by transforming UR3148-1B with a *URA3*-linked *PAP1*-integrating plasmid, selecting for 5-fluoroorotic acid resistance (indicating integrant loss), and screening for temperature resistance at 37°. To test the ability of previously described *RET1* alleles to suppress *pap1-1*, we transformed strain 1BTH-3D with plasmids bearing cold-sensitive alleles of *RET1* (cf. Table 2). Resulting strains were then transformed with a DNA fragment bearing a *ret1::HIS3* disruption to replace the chromosomal *RET1* copy. Plasmids were rescued from His+ transformants, and we determined that cold-resistant strains were the result of disruptions of the plasmid-borne *ret1* mutant alleles, whereas cold-sensitive strains yielded plasmids with *ret1* intact.

For the suppressor isolation, 50 individual colonies (~1 × 10^7 cells/colony) of UR3148-1B grown on YEPD agar medium at 25° were resuspended in 0.5 ml YEPD broth and 0.1 ml aliquots were spread onto independent plates incubated at 30°. After 5 days, 10–50 revertants/plate were picked and patched onto fresh plates incubated at 14°, 25°, 30°, and 37°. We backcrossed strains that grew at 37° to A364A and were unable to isolate temperature-sensitive segregants from the progeny of that diploid, indicating that growth at 37° was due to intragenic reversion of *pap1-1*. Five strains were isolated that grew at 30°, but not at 37°, that also exhibited slow growth at 14° (cf. Figure 1A).

Characterizations of suppression requiring growth in liquid media were performed at 25°, or after shift to 14° or 30° for times indicated, in either YEPD or synthetic complete media (SHERMAN *et al.* 1986) lacking uracil (to maintain plasmids). Suppression on plates (Figure 1) was tested at 29° due to a slightly better contrast between growth of the *pap1-1* mutant and suppressors at that temperature on solid media. Genetic techniques were performed as described by SHERMAN *et al.* (1986) and ROTHSTEIN (1991). Transformation of yeast was performed by lithium acetate as described by SCHIESTL and GEITZ (1989). *Escherichia coli* strain DH5a was used for all recombinant DNA manipulations.

TABLE 2

Plasmids and oligonucleotides

Plasmid/oligonucleotide	Relevant characteristics or sequence	Reference
YCplac33	*CEN4, URA3,* library cloning vector	GEITZ and SUGINO (1988)
YIplac211	Integrative, *URA3,* linkage mapping vector	GEITZ and SUGINO (1988)
YEp24	Episomal, *URA3*	BOTSTEIN *et al.* (1979)
YCpXH	*CEN4, URA3,* containing *PAP1* open reading frame	PATEL and BUTLER (1992)
pC1	*CEN4, URA3,* containing *RET1* open reading frame	This study
pC1ΔSst	*CEN4, URA3,* containing an incomplete *RET1* open reading frame	This study
pSA23	*CEN3, LEU2, CYH2, RET1*	NICK ZECHERLE
pBK-I11	*CEN3, TRP1,* containing *ret1-510*	SHAABAN *et al.* (1995)
pBK-I15	*CEN3, TRP1,* containing *ret1-515*	SHAABAN *et al.* (1995)
pSA11	Plasmid source of fragment containing Δ*ret1,* disrupted by *HIS3*	SHAABAN *et al.* (1995)
o5.8S	5′-TGCGTTCTTCATCGATGCGAGAACC-3′	SKRYABIN *et al.* (1984)
o5S	5′-CAGTTGATCGGACGGGAAAC-3′	VALENZUELA *et al.* (1977)
otRNA^{Tyr}	5′-CCAACTTGGCTACCGAGAG-3′	KOHRER *et al.* (1990)

Plasmids and oligonucleotides: The plasmids and deoxyoligonucleotides utilized in this report are described in Table 2. Restriction enzymes were purchased from Gibco-BRL, Promega, or New England Biolabs, and digestions were performed as per manufacturers' instructions. Double-stranded DNA probe templates were prepared by diethylaminoethyl paper purification from 1% agarose gels and labeled by random hexamer priming with [5′ a^{32}P] deoxy CTP and the Klenow fragment of DNA polymerase (Boehringer-Mannheim), according to the manufacturers' instructions. All deoxyoligonucleotides were synthesized by Oligos Etc., Inc. Deoxyoligonucleotide probes were prepared by T4 kinase (Gibco-BRL) labeling of 25 pmol deoxyoligonucleotide with [5′ a^{32}P] ATP, according to the manufacturers' instructions. All probes were purified through G-25 Sephadex.

RNA analyses: Total RNA was prepared and Northern analysis carried out as described by PATEL and BUTLER (1992). The amount of a particular mRNA was quantitated by storage phosphorimager analysis (Molecular Dynamics) and normalized to rRNA that was quantitated by fluorimager analysis (Molecular Dynamics) of ethidium bromide-stained gels or by storage phosphorimager analysis of 5′^{32}P-labeled deoxyoligonucleotide probes hybridized to 5.8S rRNA (cf. Table 2), using Molecular Dynamics Imagequant software.

Total *in vivo* poly(A) content determinations were performed as described by PROWELLER and BUTLER (1994).

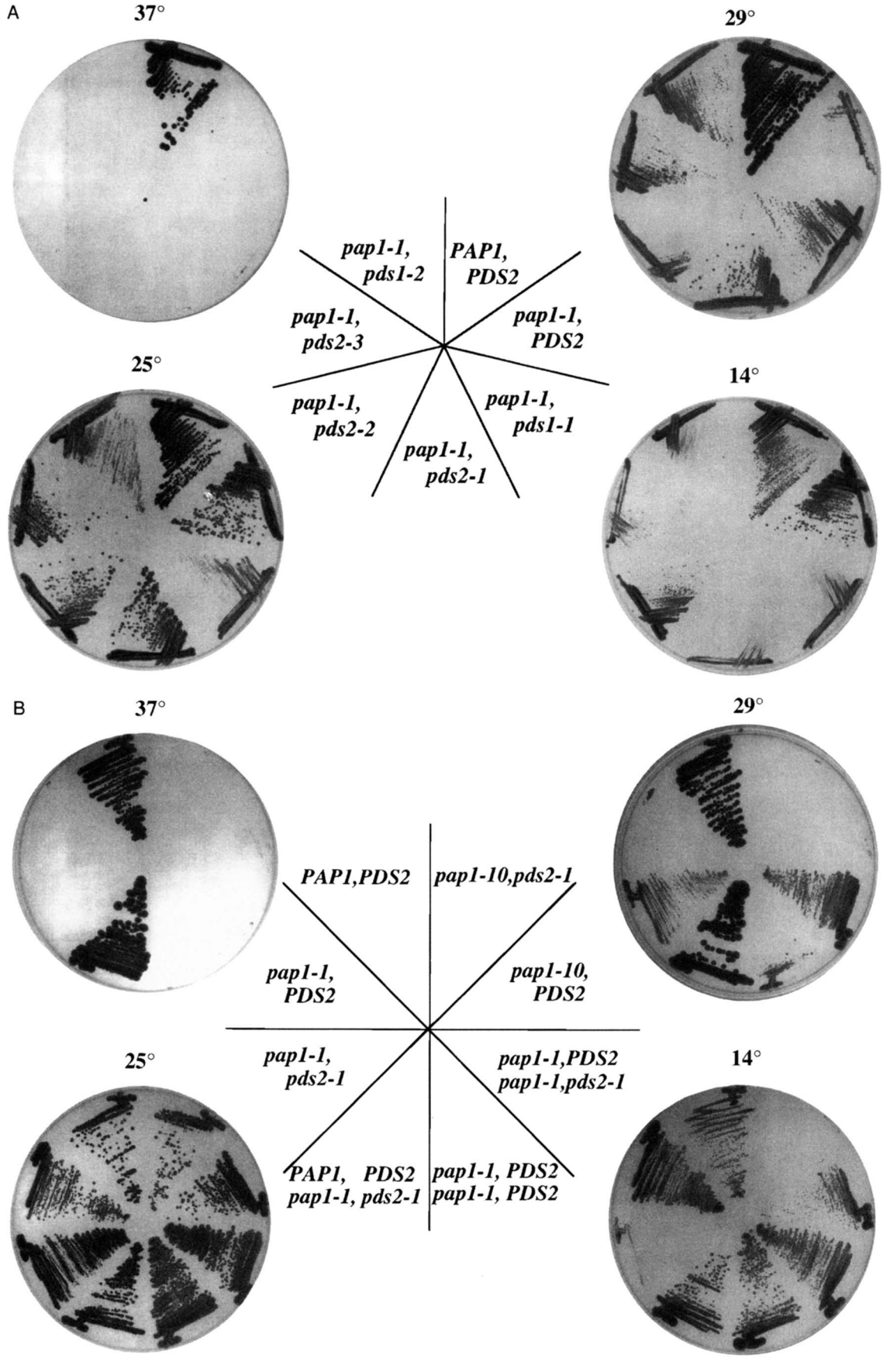
The protocol for mRNA decay measurements has been described (HERRICK *et al.* 1990). Cells were grown at 25° to mid-log phase and split into 25° and 30° subcultures. After 6 hr postshift, roughly 1.5 generations, Thiolutin (a kind gift of Dr. SAUL B. KADIN, Pfizer) was added to each culture from a 2 mg/ml stock dissolved in dimethylsulfoxide to a final concentration of 4 mg/ml. Aliquots of 10^8–10^9 cells were harvested at time of addition of Thiolutin and at 10-min intervals thereafter. Total RNA was analyzed by Northern blotting, and blots were exposed to Kodak XAR film or to a phosphorimaging screen (Molecular Dynamics) for quantitation.

For analyses of RNA polymerase III transcripts, 3 mg of total RNA were separated on an 8% polyacrylamide, 8 M urea gel, and RNAs were transferred to Gene Screen membranes (DuPont) by electroblotting at 10 V for 16 hr at 4°. Hybridizations were performed under aqueous conditions using oligonucleotide probes shown in Table 2, and blots were exposed to film or a phosphorimaging cassette for quantitation.

Library construction and isolation of PDS2: We constructed our library essentially as described by ROSE and BROACH (1991). Genomic DNA (1 mg) prepared from S288C was partially digested with *Sau*3AI at a concentration of 10 U/mg for 1 hr at 37°, and size fractionated on sucrose density gradients. DNA (10–15 kb) was pooled and ligated to *Bam*HI-digested, dephosphorylated YCplac33 for 24 hr at 14° at a final concentration of 20 mg/ml and a molar ratio of 1:4, vector to insert. Ligations were transformed by electroporation into *E. coli* strain DH5a, yielding 20 transformants per ng ligated DNA. White and light blue colonies (indicating inserts) were observed on LB Amp. + X-gal plates at a frequency of 80%. Random analysis of plasmid DNA isolated from 20 white or light blue colonies revealed that all had inserts ranging in size from 7 to 15 kb. Eleven thousand independent transformants were pooled, representing four to five genome equivalents of yeast DNA inserts. Cesium chloride-purified DNA was prepared from this stock and transformed into UR3148-1B to check yeast gene representation. Fifty-eight of 60,000 transformants demonstrated complementation of the *pap1-1* mutation, and 54 demonstrated complementation of *lys2,* indicating a representation for single-copy nuclear genes (*PAP* and *LYS2*) of ~0.1%. Twenty milligrams of library DNA was transformed into *pds2-1* strain ABC2-2D, and plates were incubated at 25° for 36 hr and shifted to 14° until putative *PDS2*-containing complementing plasmid-bearing strains grew above background levels. Plasmid DNA was rescued from yeast cells by glass bead disruption and retransformed into ABC2-2D to confirm plasmid linkage to complementation. For linkage analysis, complementing plasmid pC1 was digested with *Sst*I, and the resulting 500 nucleotide *RET1*-specific fragment was purified from a 1% agarose gel by diethylaminoethyl paper purification and ligated to *Sst*I-digested, phosphatase-treated YIplac211. The resulting plasmid, YIpRETSst, was digested within the *RET1* fragment with *Bgl*II, transformed into strain ABC2-2D and analyzed as described in the text.

RESULTS

Identification and characterization of polyadenylation defect suppressors (*PDS*): We undertook a pseu-


A
37°
29°
pap1-1, pds1-2
PAP1, PDS2
pap1-1, pds2-3
pap1-1, PDS2
25°
14°
pap1-1, pds2-2
pap1-1, pds1-1
pap1-1, pds2-1
B
37°
29°
PAP1,PDS2
pap1-10,pds2-1
pap1-1, PDS2
pap1-10, PDS2
25°
14°
pap1-1, pds2-1
pap1-1,PDS2
pap1-1,pds2-1
PAP1, PDS2
pap1-1, pds2-1
pap1-1, PDS2
pap1-1, PDS2

doreversion analysis of the previously described *pap1-1* temperature-sensitive mutation in *S. cerevisiae,* in an effort to identify genes whose products interact physically or functionally with poly(A) polymerase. In this analysis, we limited to further study those strains that had acquired a conditional cold-sensitive phenotype of their own. Our rationale for this bias was twofold. First, for practical reasons, we wanted an independent phenotype to allow characterization of suppressors independently of the *pap1-1* mutation. Second, in an effort to identify gene products specifically involved in 3′ end formation, we hoped for extragenic suppression of our conditional mutation to occur through proteins that functionally interact with Pap1-1 protein to restore activity at high temperature. This strategy has been employed successfully in analyses of phage morphogenesis (JARVIK and BOTSTEIN 1975), yeast actin polymerization (NOVICK *et al.* 1989), and the yeast cell division cycle (MOIR *et al.* 1989). We isolated five suppressors from an initial population of about 5000 spontaneously arising pseudorevertants that allow growth at the restrictive temperature of 30° and exhibit a cold-sensitive, slow growth phenotype at 14° (Figure 1A). Note that no suppressor can grow at 37°; we found only intragenic revertants arising at that temperature. We backcrossed each of these strains to the normal progenitor of the *pap1-1* mutant and found that the temperature-sensitive and cold-sensitive phenotypes segregated independently of one another, and that the suppression always associated with cold-sensitive growth. The cold-sensitive phenotype also demonstrated Mendelian 2:2 segregation, indicating that each suppressor arose from a mutation in a single-copy nuclear gene. Crosses of cold-sensitive segregants to each of the suppressor strains revealed that three of the five mutations represent a single complementation group (*PDS2*).

Based on its representation in our suppressor screen, we characterized further the *PDS2* complementation group. We crossed a *pap1-1, pds2-1* strain to both *PAP1, PDS2* and *pap1-1, PDS2* strains and found that the cold-sensitive phenotype of *pds2-1* is recessive in the presence of both *PAP1* and *pap1-1* alleles (Figure 1B). On the other hand, in a homozygous *pap1-1* diploid the suppressor phenotype of *pds2-1* is dominant to wild type (Figure 1B). Since we have shown *pap1-1* to be a loss of function mutation, the dominant suppression of this defect suggests a gain of function of poly(A) polymerase activity due to the *pds2-1* mutation and implies a potentially important interaction between the two genes' products. Allele-specific suppression provides further genetic evidence for such an interaction. We crossed a *pds2-1* strain with a strain harboring an allele of *PAP1*

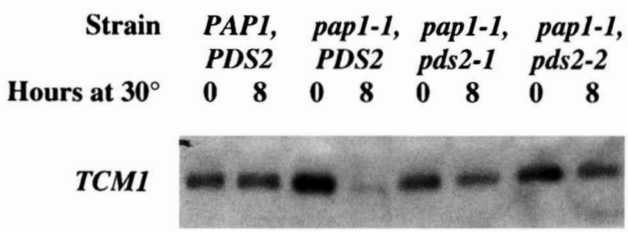


FIGURE 2.—Steady-state mRNA levels in *PAP1, pap1-1,* and *pap1-1, pds2* strains at permissive and restrictive temperatures. Cells were grown at 25°, and at time zero the culture was split and half remained at 25° while the other half was incubated at 30°. Cells were harvested 8 hr later and total RNA was prepared. mRNA levels were determined by Northern blot analysis.

(herein named *pap1-10*) independently isolated from the same collection of temperature-sensitive yeast mutants as *pap1-1* (HARTWELL 1967; WINEY *et al.* 1991), and we detected no suppression of the temperature-sensitive phenotype (Figure 1B). Therefore, the recessive *pds2-1* mutation suppresses at least one, but not all, mutant alleles of *PAP1* in a dominant fashion.

The *pds2-1* mutation restores steady state levels of various mRNAs *in vivo* at 30°: The physiological effects of the *pap1-1* lesion on yeast mRNA abundance and translatability have been described previously by our laboratory (PATEL and BUTLER 1992; PROWELLER and BUTLER 1994). Our work led to the conclusion that the lethal physiological effect associated with this mutation is the inability of the cell to accumulate certain mRNAs. We expected, then, that our *pds2* suppressor strains might demonstrate enhanced accumulation of mRNAs relative to *pap1-1* cells. Comparison of mRNA levels in wild type, *pap1-1* and two of the *pds2* suppressor strains by Northern analysis of total RNA prepared before and after shift to the nonpermissive temperature of 30° showed a significant decrease in the steady-state concentration of *TCM1* mRNA in *pap1-1* cells that was restored to near normal levels in each *pds2* strain at 30° (Figure 2). We then fractionated the 8-hr 30° shifted RNAs described above by oligo-dT cellulose chromatography and analyzed the bound and unbound fractions by Northern blotting. As expected from the results of previous work, at high temperature *pap1-1* cells do not produce *TCM1* or *ACT1* mRNA that hybridize to oligo-dT. Conversely, a substantial proportion of these messages from each of the suppressors does bind to oligo-dT (data not shown). Therefore, poly(A) tails of at least moderate length (20 As or more) (GRONER *et al.* 1974) are present at restrictive temperature in each of our suppressor strains, at least for the particular messages we examined.

The *pds2-1* mutation suppresses the polyadenylation

FIGURE 1.—Growth characteristics of *pap1-1* and *pap1-1, pds-* strains. (A) Partial suppression of the temperature sensitivity of growth associated with the *pap1-1* mutation by cold-sensitive *pds* mutants. (B) Genetic analysis of *pds2-1.* Positions of haploid and diploid *pap1-1* and *pds2-1*-bearing strains are indicated in the diagram, as well as *pap1-10* and *pap1-10, pds2-1.* Positions of wild-type, *pap1-1* and suppressor strains are indicated in the diagram. Plates were grown at 37° for 3 days, 29° for 7 days, 25° for 3–4 days, and 14° for 7–10 days.

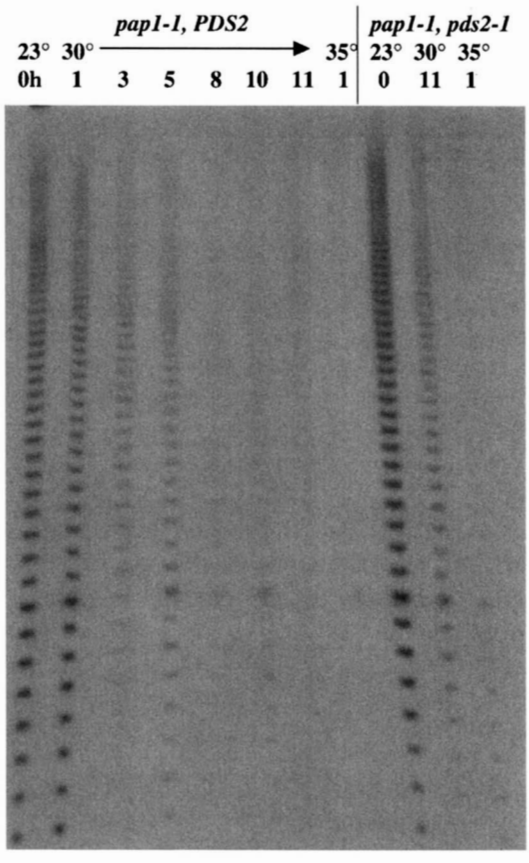


FIGURE 3.—Poly(A) content of *pap1-1* and *pap1-1, pds2-1* strains at permissive and restrictive temperatures. *pap1-1* or *pap1-1, pds2-1* strains were grown at 25° and shifted to either 30° or 35°. Total RNA was prepared at indicated times, and equal amounts were 3′ end labeled with RNA ligase and [5′-^{32}P]pCp. Labeled RNAs were then hydrolyzed with RNases A and T1, and poly(A) tracts were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography (PROWELLER and BUTLER 1994).

defect of *pap1-1 in vivo:* The defect in the polyadenylation step of mRNA 3′ end formation at high temperature in *pap1-1* cells leads to a rapid loss of poly(A) tails *in vivo* (PROWELLER and BUTLER 1994). We showed in the previous section that *pds2-1* leads to an increase in the ratio of poly(A)+ to poly(A)− *TCM1* and *ACT1* mRNAs. To test whether the *pds2-1* mutation might restore total cellular poly(A) content at the restrictive temperature for *pap1-1* cells, we examined total poly(A) content *in vivo* in *pap1-1* and *pap1-1, pds2-1* strains at permissive and restrictive temperatures (Figure 3). Eight hours after shift to 30°, *pap1-1* cells have lost most of the poly(A) in the cell, while *pap1-1, pds2-1* cells exhibit partially restored levels of poly(A) of normal length, even after 11 hr at 30°. At 35°, a temperature too severe for *pds2-1* to suppress the *pap1-1* growth defect (cf. Figure 1A), RNA from the double mutant exhibits a lack of poly(A) similar to that seen in RNA from the *pap1-1* mutant. As noted in the previous section, the accumulation of poly(A)+ mRNA by *pds2-1* suggests a restoration of polyadenylation activity. Alternatively, suppression could result from slowed decay of the small amount of polyadenylated mRNA found in *pap1-1* cells at restrictive temperature.

The *pds2-1* mutation does not increase the stability of mRNA *in vivo:* To determine directly whether *pds2-1* slows the decay of mRNA, we utilized the global transcriptional inhibitor Thiolutin (TIPPER 1973; HERRICK *et al.* 1990) in an analysis of mRNA decay rates between isogenic *PAP1, PDS; pap1-1, PDS;* and *pap1-1, pds2-1* strains (Figure 4A). Individual mRNA half-lives were determined by measuring the concentration of each mRNA over time after inhibition of RNA polymerase with Thiolutin and normalizing to the stable, RNA polymerase I-transcribed ribosomal RNAs (Figure 4B). We observed no significant difference in the half-life of *TCM1* between the three strains, both before and after shift to 30°. Since the steady-state concentration of *TCM1* is reduced in *pap1-1* cells after shift to 30° relative to wild type, but there is no change in its cytoplasmic rate of turnover, we deduce that the *pap1-1* mutation leads to an inability to accumulate mature message (see DISCUSSION). *pap1, pds2-1* strains, however, do accumulate *TCM1* but not by affecting its rate of turnover. Furthermore, *rp29* mRNA becomes less stable in the presence of the suppressor mutation, suggesting feedback regulation of ribosomal protein mRNA concentrations independent of poly(A) status. Nonribosomal protein-encoding mRNAs, such as *PAP1, RET1,* and *CLN3,* were also examined and demonstrated no increase in stability (data not shown). The fact that steady-state mRNA concentrations approach wild-type levels in *pap1-1, pds2-1* cells but their rates of decay are unchanged or higher suggests that the rate of productive synthesis of these messages has increased. This conclusion is consistent with poly(A) polymerase's role in the synthesis of stable mRNA and implies that *pds2-1* suppresses the defect in mRNA synthesis associated with the *pap1-1* mutation.

Cloning of *PDS2:* The recessivity of the *pds2-1* cold-sensitive phenotype allowed us to clone the wild-type gene by complementation. We transformed a *PAP1, pds2-1* strain with a centromere-based library, screened for transformants that grew better than background at 14°, and found three complementing plasmids (pC1, pC5, and pC23; Figure 5). Each plasmid was rescued from yeast and retransformed, confirming plasmid linkage to complementation. We sequenced into the pC1 insert and found 100% identity to a region on chromosome *XV* encompassing *MGM1, PTP2, RET1,* and an uncharacterized open reading frame (JAMES *et al.* 1991; JAMES *et al.* 1992). Due to the large insert size of pC23, we could not be sure which gene was the true cognate counterpart of *pds2-1,* and therefore created two deletions to make that determination (Figure 5). pC1DSst removed a small C-terminal portion of *RET1,* and pC5DMlu deleted *RET1* and *PTP2.* We then transformed these constructs back into *pds2-1* strains and found that only those plasmids carrying intact *RET1* could complement the cold sensitivity. We subcloned

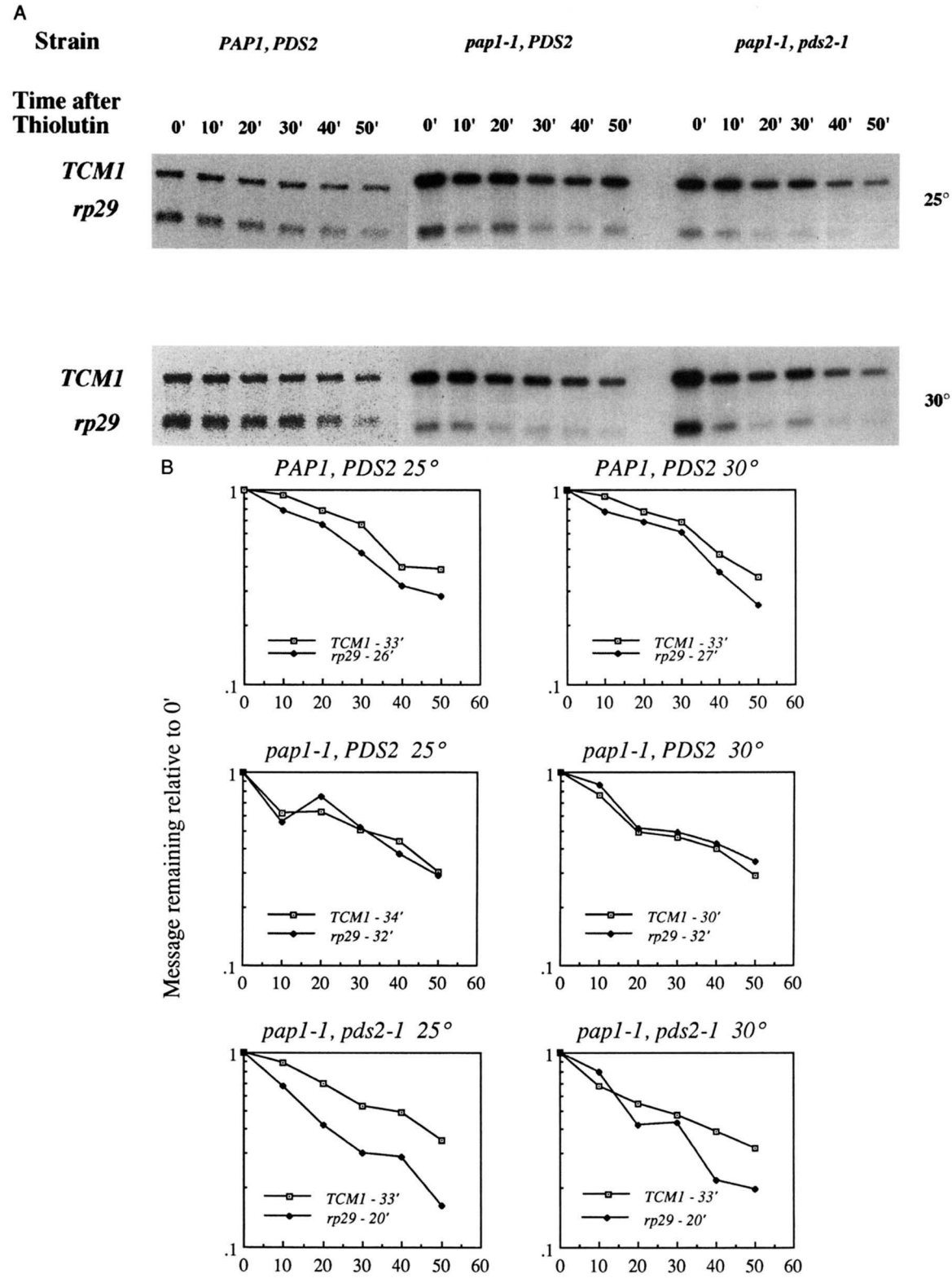


FIGURE 4.—Comparison of individual mRNA decay rates in wild-type, *pap1-1*, and *pap1-1, pds2-1* strains at permissive and restrictive temperatures. (A) Wild-type, *pap1-1*, and *pap1-1, pds2-1* strains were grown at 25° and split to 25° and 30° and allowed to grow for 6 hr. Thiolutin (2 mg/ml) was added to equal numbers of cells to a final concentration of 4 mg/ml at time zero and total RNA was prepared at indicated times. mRNA levels were determined by Northern blot analysis and normalized to 18S rRNA. (B) Quantitation of *TCM1* and *rp29* mRNAs' rates of decay. Normalized mRNA concentrations were plotted as the abundance relative to time zero as a function of time after transcription shut-off. The indicated mRNA half-lives were determined from the slopes of these plots (HERRICK *et al.* 1990).

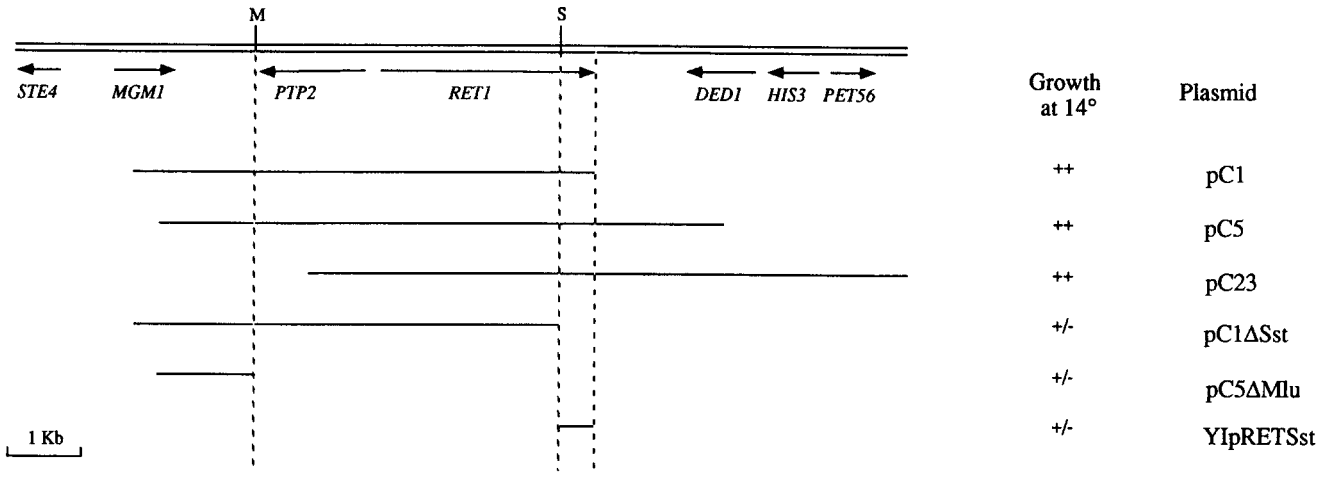


FIGURE 5.—Complementation analysis of subclones of the *RET1* region. The region of chromosome *XV* containing *RET1* and nearby coding regions is depicted in the figure. The lines below represent the restriction fragments of complementing library plasmids or the plasmid construct subclones indicated at the right. Plasmid pC1DSst was constructed by deletion of a *RET1*-specific 3′-terminal *Sst*I fragment from sites at nucleotide 3785 within the *RET1*-coding region and the multicloning site of pC1. Plasmid pC5DMlu resulted from deletion of a fragment flanked by the *Mlu*I site within *PTP2* and a *Sal*I site in the multicloning site of pC5. YIpRETSst was constructed by ligating the *Sst*I fragment from pC1 into *Sst*I-digested YIplac211. Plasmids were transformed into a *pds2-1* strain and complementation of the recessive cold-sensitive phenotype was determined by the ability of the transformed cells to grow normally at 14°. S, *Sst*I; M, *Mlu*I.

the 500-bp C-terminal *Sst*I fragment from *RET1* into the *Sst*I site of YIplac211 (Figure 5) to confirm linkage of *pds2-1* to the chromosomal locus of *RET1*. YIpRETSst was linearized within the *RET1* fragment and transformed into *pds2-1* strain ABC2-2D. Integrants still demonstrated cold sensitivity and were crossed to a normal strain. Upon sporulation and dissection of 29 tetrads, we observed germination of 20 complete tetrads, revealing 19 parental ditype and one tetratype, which indicates tight linkage of the integrated YIpRETSst and *pds2-1*. From the linkage and deletion/complementation analyses, we conclude that a mutation in the essential gene *RET1*, encoding the second largest subunit of RNA polymerase III, suppresses the *pap1-1* defect.

We showed above that *pds2-1* exerts allele-specific suppression over *pap1-1*. In a *pap1-1* background, we replaced the normal copy of *RET1* with previously described cold-sensitive alleles (SHAABAN *et al.* 1995, provided by Dr. N. ZECHERLE, see MATERIALS AND METHODS) and found that these alleles were unable to suppress *pap1-1* (Table 3) in either dominant or rececessive fashion. It is noteworthy that these previously described *ret1* mutations were mapped to the very C-terminus of Ret1, within the terminal 560 bp distal to the *Sst*I site. We have not sequenced the *pds2-1* mutation, but it cannot map to this region based on our integrative linkage analysis described above. This indicates that we have uncovered mutations in a particular portion of Ret1 that is functionally associated with Pap1.

The *pds2-1* mutation causes alterations in RNA polymerase III transcription: RNA polymerase III transcribes 5S rRNA, tRNAs and various other small RNAs (GEIDUSCHEK and TOCCHINI-VALENTINI 1988; MOSRIN and THURIAUX 1990; WILLIS 1993). Thus, we expected defects in the synthesis of one or all of these RNAs to be associated with the *pds2-1* mutation. We prepared total RNA from isogenic *PDS2* and *pds2-1* strains in *PAP1* and *pap1-1* backgrounds after growth at 25° and subsequent shift to either 14° or 30° for one to two generations and compared steady-state levels of small RNAs at low and high temperatures by Northern analysis (Figure 6). The relative amounts of these RNAs were normalized to the RNA polymerase I 5.8S rRNA transcript and compared to wild type (Table 4). We did not observe an appreciable fluctuation of 5S rRNA steady-state levels, although the *pds2-1* mutation causes the appearance of a 5S rRNA doublet at 14° in cells containing the mutation. Other Pol III transcripts whose steady-state levels are not significantly affected by the *pds2-1* mutation include *NME1*, the RNA component of RNase MRP; *SCR1*, the RNA component of the yeast signal recognition particle; and *SNR6*, an RNA integral to mRNA splicing (data not shown). Steady-state levels of $tRNA^{Tyr}$, conversely, are reduced 30–40% of normal levels at 14° in *pap1-1, pds2-1* cells. Qualitative observations of ethidium bromide-stained RNA gels suggest a general tRNA accumulation defect at low temperature (data not shown). Interestingly, though the alteration of mature 5S rRNA transcribed at low temperature is a recessive defect associated with *pds2-1*, the reduction in tRNA concentration is dominant to the wild-type copy of *PDS2* present on plasmid pC1. It is unlikely that these fluctuations in tRNA concentrations are important for the mechanism of suppression though, since we do not detect any variation between mutant and suppressor strains at high temperature (Table 4). If suppression is due to a product of misregulated RNA polymerase III transcription, then the expression of this as yet uncharacterized

TABLE 3

Allele specificity of *pds2-1* suppression

Strain	Genotype	Growth at 14°	Growth at 25°	Growth at 30°
1BTH-3D	*pap1-1*	++	++	−
UR3148-1BC2	*pap1-1, pds2-1*	+/−	++	+
UR3148-1BC2:pC1	*pap1-1, pds2-1/PDS2*	++	++	+
SAI11	*PAP1, ret1-510*	+/−	++	+++
1BTHI111 Δret	*pap1-1, ret1-510*	+/−	++	−
1BTHI11	*pap1-1, ret1-510/RET1*	++	++	−
SAI15	*PAP1, ret1-515*	+/−	++	+++
1BTHI15 Δret	*pap1-1, ret1-515*	+/−	++	−
1BTHI15	*pap1-1, ret1-515/RET1*	++	++	−

Strain derivatives of 1BTH-3D are isogenic, except for the *RET1* allele present. Plasmids bearing mutant *ret1* alleles (cf. Table 2) were transformed into 1BTH-3D to produce strains 1BTHI11 and 1BTHI15, which are hemizygous at their *RET1* locus. The chromosomal copy of *RET1* was disrupted to produce strains 1BTHI11 Δret and 1BTHI15 Δret (see MATERIALS AND METHODS). Growth on plates was scored as in the legend to Figure 1A.

acterized transcript involved in mRNA 3′ end formation would presumably be affected in a dominant fashion at 30° by the *pds2-1* mutation. We have not detected variations of any previously described yeast RNA polymerase III transcript between *pap1-1* and *pap1-1, pds2-1* cells at 30° but cannot rule out the possibility that such an RNA exists.

DISCUSSION

We have presented the results of a suppressor analysis of a yeast polyadenylation mutation, *pap1-1*. This mutation causes a temperature-sensitive lethal defect in mRNA 3′ end processing that results in the synthesis of poly(A)− mRNAs (PATEL and BUTLER 1992). A portion of these poly(A)− mRNAs accumulates and under-

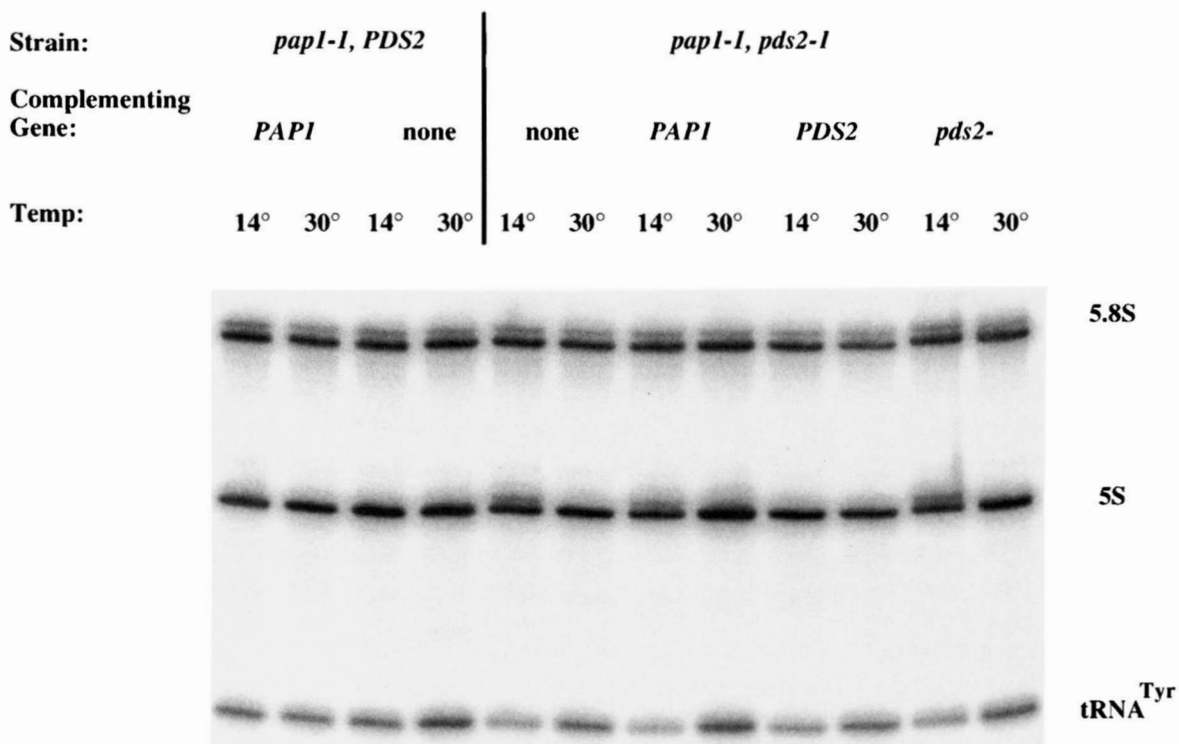


FIGURE 6.—Analysis of small RNAs transcribed by RNA polymerase III in various genetic backgrounds at low and high temperatures. A *pap1-1* strain was transformed with plasmids YCpXH or YEp24 (indicated as complementing gene: *PAP1* or none, respectively) and a *pap1-1, pds2-1* strain was transformed with plasmids YCplac33, YCpXH, pC1, or pC1DSst (indicated as complementing gene: none, *PAP1, PDS2,* or *pds2-*, respectively, cf. Figure 5). These transformed strains were grown in selective media at 25° and split to either 14° for 24 hours or 30° for 6 hr. Equal numbers of cells were harvested and total RNA was prepared. Three milligrams of each RNA was separated by denaturing 8% polyacrylamide, 8 M urea gel electrophoresis and transferred to nitrocellulose by electroblotting for quantitation (cf. Table 4).

TABLE 4

Analysis of RNAs transcribed by RNA Polymerase III

Genotype	$tRNA^{Tyr}$/5.8S rRNA			5S/5.8S rRNA			5S doublet
	14°	30°	14°/30°	14°	30°	14°/30°	
pap1-1/*PAP1*	1.0	1.0	1.0	0.9	1.0	0.9	–
pap1-1	1.0	1.6	0.6	0.9	1.1	0.8	–
pap1-1, pds2-1	0.7	1.6	0.4	1.1	1.0	1.1	+
pap1-1/*PAP1*, *pds2-1*	0.7	1.5	0.5	0.9	1.0	0.9	+
pap1-1, *pds2-1*/*PDS2*	0.8	1.5	0.5	1.1	1.0	1.1	–
pap1-1, *pds2-1*/*pds2-*	0.6	1.6	0.4	0.9	1.1	0.8	+

Steady-state RNA levels of 5S rRNA and $tRNA^{Tyr}$ were normalized to 5.8S rRNA and are presented in the table relative to wild-type levels at 30°. The data shown represent the averages of two or three determinations.

goes translation while other mRNAs fail to reach appreciable steady-state levels (PROWELLER and BUTLER 1994). Characterization of one of the *pap1-1* suppressors, *pds2-1*, revealed it to be an allele of *RET1*, encoding the second-largest subunit of RNA polymerase III. This surprising finding led us to consider whether the *PDS2* suppressors might represent a class of pleiotropic suppressors of RNA synthesis defects, such as *prp20* and *rna1-1* (HOPPER *et al.* 1978; AEBI *et al.* 1990a). Three observations argue against this mechanism of suppression. First, *pds2-1*, while recessive on its own, is a dominant suppressor of *pap1-1*. Second, *pds2-1*, but not other alleles of *RET1*, suppresses the *pap1-1* mutation in an allele-specific fashion. Third, *PDS2* alleles represent the most common class of suppressor of *pap1-1* isolated in this screen. The fact that *pds2-1* exerts a dominant allele-specific effect on the *pap1-1* mutation suggests that poly(A) polymerase and subunit B of RNA polymerase III interact directly, or that they interact through their products in a common pathway.

Dominant allele-specific suppression sometimes indicates direct physical interaction between two gene products. Such an interpretation would implicate subunit B of RNA polymerase III as a factor directly involved in mRNA 3′ end processing in *S. cerevisiae*. We consider this possibility unlikely merely because no precedent exists for the sharing of RNA polymerase subunits except among the three eukaryotic RNA polymerases. It has been reported that functional mRNAs can be transcribed by RNA polymerase III (GUNNERY and MATHEWS 1995) and in some cases carry a poly(A) tail (CARLSON and ROSS 1983), but these observations have been disputed (SISODIA *et al.* 1987). To add to the controversy, it has also been demonstrated that murine tumor cells express natural RNA polymerase III transcripts that are polyadenylated (KRAMEROV *et al.* 1990). Taken together, these data suggest the possibility that RNA polymerase III may interact functionally with the mRNA 3′ end processing machinery, if not acting as an integral subunit. Furthermore, previous characterization of *RET1* mutant alleles indicated that this subunit is directly involved in the termination of RNA polymerase III transcription (JAMES and HALL 1990; JAMES *et al.* 1991; SHAABAN *et al.* 1995). A role for Ret1 in enhancing RNA polymerase II 3′ end formation is not inconceivable then, as this activity could represent an evolutionary remnant of the role of this protein in general RNA 3′ end formation. We investigated the possibility of a physical interaction between Ret1 and Pap1 by using the yeast two-hybrid system (FIELDS and SONG 1989) but found no evidence for such an interaction (data not shown).

Suppression of *pap1-1* by alleles of *RET1* could occur through RNA polymerase III synthesis of an RNA involved in mRNA 3′ end formation. Dominant suppressor mutations might be preferred in this case since the gain of function, implied by the dominant nature of the mutation, could increase the rate of synthesis of the RNA product in question, thus driving the reaction requiring it toward product formation. Investigation of the effect of the *pds2-1* mutation on the synthesis of products of each class of RNA polymerase III promoter showed effects on both class I (5S rRNA) and class II (tRNA). The yeast class II RNAs *SCR1*, *NME1*, and *SNR6*, which represent a third class of promoters in higher eukaryotes (WILLIS 1993), were unaffected by *pds2-1*. The *pds2-1* mutation does not cause variation in $tRNA^{Tyr}$ levels at 30° in a *pap1-1* background, therefore a model to explain suppression that requires the direct participation of tRNAs in mRNA 3′ end formation seems unlikely. Furthermore, increased synthesis by a mutant RNA polymerase III of an informational sup-

pressor that could conceivably increase poly(A) polymerase activity by altering the amino acid sequence of the mutant protein also seems unlikely, since we observed no suppression by *pds2-1* of a variety of other unrelated mutations used as genetic markers in our strains (data not shown).

Suppression of the *pap1-1* defect by an increase in the synthesis of an RNA component of the polyadenylation machinery, or by a mutation affecting the ability of Ret1 to interact with Pap1, should suppress the polyadenylation defect caused by *pap1-1 in vitro*. Unfortunately, cell-free polyadenylation extracts derived from strains carrying temperature-sensitive alleles of *PAP1* have very low levels of poly(A) polymerase activity (PATEL and BUTLER 1992; MINVIELLE-SEBASTIA *et al.* 1994). We have used the system to determine the effect of *pds2-1* on the thermostability of the rate of polyadenylation *in vitro* but have failed to produce convincing results supporting a role in this step (data not shown). Also, we have examined the steady-state poly(A) content of *PAP1, pds2-1* strains *in vivo* at various temperatures but have failed to produce evidence that *pds2-1* causes a significant defect by itself (data not shown). We are aware that this does not lend support to our proposed involvement of *RET1* in mRNA 3′ end formation. The observed allele specificity of suppression may suggest, though, that the involvement of *RET1* in this process is only detectable in a compromised *pap1-1* background.

Polyadenylation remains the only mRNA processing reaction for which the role of an RNA component has not been demonstrated. Despite early experiments indicating a requirement for a small RNA in polyadenylation site cleavage (MOORE and SHARP 1985; HASHIMOTO and STEITZ 1986; CHRISTOFORI and KELLER 1988), reconstitution of cleavage and polyadenylation reactions *in vitro* with extensively purified components provided no evidence for the participation of an RNA component (TAKAGAKI *et al.* 1989; BIENROTH *et al.* 1991). Although we do not favor a small RNA as an explanation for our findings, we note that recent studies provide evidence for a role for U1 snRNP in enhancing polyadenylation efficiency (LUTZ and ALWINE 1994; LUTZ *et al.* 1996) Biochemical characterization of mRNA 3′ end processing in yeast has not advanced far enough to reach a conclusion about the requirement for an RNA component. The mechanism of polyadenylation in yeast and the factors characterized so far appear similar to those characterized from humans. However, differences will surely emerge as work from the yeast system advances.

Slowing the rate of mRNA decay represents a possible way to suppress a polyadenylation mutation, since many mRNAs fail to accumulate soon after polyadenylation shut-off in *pap1-1* strains (PROWELLER and BUTLER 1994). For instance, suppression of certain nonsense and frameshift mutations can result from inactivation of the Upf1 pathway, which increases the half-life of these mutant mRNAs (LEEDS *et al.* 1990). Since translational elongation appears to play a role in mRNA turnover and because a tRNA nucleotidyltransferase mutation stabilizes mRNAs by slowing protein synthesis (AEBI *et al.* 1990b; PELTZ *et al.* 1992), we considered whether a defect in RNA polymerase III might increase mRNA levels indirectly by decreasing charged tRNA levels. Measurements of mRNA turnover rates in *pap1-1, pds2-1* cells indicated, however, that *pds2-1* does not increase the half-lives of the mRNAs tested. One of the transcripts we tested, *TCM1,* previously showed a threefold increase in half-life due to inactivation of tRNA nucleotidyltransferase, demonstrating the sensitivity of its rate of turnover to changes in tRNA levels (PELTZ *et al.* 1992). Thus, it does not appear that the RNA polymerase III defect caused by the *pds2-1* mutation suppresses *pap1-1* by stabilizing mRNAs through reduced tRNA levels. Indeed, a deletion of *XRN1,* which encodes a key enzymatic activity in the decay pathway of many normal mRNAs, fails to suppress *pap1-1,* providing circumstantial evidence that mutations that slow cytoplasmic mRNA decay do not suppress *pap1-1* (A. PROWELLER, unpublished observations).

The increase in the steady-state levels of poly(A)+ mRNA caused by *pds2-1* in the absence of an increase in mRNA half-life argues that the suppressor acts to increase the rate of synthesis of stable mRNA. Defects in mRNA 3′ end formation cause the synthesis of mRNAs with abnormal 3′ ends that accumulate to lower levels than normal mRNAs (ZARET and SHERMAN 1982; PATEL and BUTLER 1992; PROWELLER and BUTLER 1994; MANDART and PARKER 1995). Suppressors could correct such defects by increasing the rate of transcription, thus raising the levels of the abnormal transcripts. A number of suppressor mutations close to the *cyc1-512* mutation probably represent suppressors of this type (KOTVAL *et al.* 1982). Alternatively, suppressors could act posttranscriptionally to increase the rate of production of mRNAs polyadenylated at their normal or nearby polyadenylation sites (GUO *et al.* 1995). This could occur by either increasing the rate of 3′ end processing or by slowing a nuclear RNA turnover pathway dedicated to the disposal of abnormally processed RNAs. The discovery that the nuclear Sut1 protein increases the halflife of *cyc1-512* mRNAs with extended 3′ ends sets a precedence for such a pathway (Z. GUO, unpublished results). In this regard, a working model for the action of *pds2* suppression of *pap1-1* polyadenylation defects would feature competing 3′ end processing and nuclear RNA degradation pathways, in which dominant *pds2* suppressors slow the degradation pathway either by synthesizing an inhibitor or by titrating away a rate-limiting component of the pathway. We believe that suppression by *pds2-1* may occur by such a mechanism, since *pds2-1* causes a significant increase in the amount of poly(A) and an increase in the ratio of poly(A)+ to poly(A)− mRNA in a *pap1-1* strain at the nonpermissive temperature. Thus, it appears that *pds2* suppressors may reveal an unprecedented connection between RNA polymerase III and the mRNA 3′ end processing machinery.

In summary, we have presented genetic and biochemical evidence linking poly(A) polymerase function to RNA polymerase III. The mechanism by which mutations in RNA polymerase III suppress a poly(A) polymerase defect remains unknown and continues as a subject of investigation in our laboratory.

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