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Isolation and Characterization of Temperature-Sensitive Mutations in *RPA190*, the Gene Encoding the Largest Subunit of RNA Polymerase I from *Saccharomyces cerevisiae*

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The isolation and characterization of temperature-sensitive mutations in RNA polymerase I from *Saccharomyces cerevisiae* are described. A plasmid carrying *RPA190*, the gene encoding the largest subunit of the enzyme, was subjected to in vitro mutagenesis with hydroxylamine. Using a plasmid shuffle screening system, five different plasmids were isolated which conferred a temperature-sensitive phenotype in haploid yeast strains carrying the disrupted chromosomal *RPA190* gene. These temperature-sensitive alleles were transferred to the chromosomal *RPA190* locus for mapping and physiology experiments. Accumulation of RNA was found to be defective in all mutant strains at the nonpermissive temperature. In addition, analysis of pulse-labeled RNA from two mutant strains at 37°C showed that the transcription of rRNA genes was decreased, while that of 5S RNA was relatively unaffected. RNA polymerase I was partially purified from several of the mutant strains grown at the nonpermissive temperature and was shown to be deficient when assayed in vitro. Fine-structure mapping and sequencing of the mutant alleles demonstrated that all five mutations were unique. The *rpa190-1* and *rpa190-5* mutations are tightly clustered in region I (S. S. Broyles and B. Moss, Proc. Natl. Acad. Sci. USA 83:3141–3145, 1986), the putative zinc-binding region that is common to all eucaryotic RNA polymerase large subunits. The *rpa190-3* mutation is located between regions III and IV, and a strain carrying it behaves as a mutant that is defective in the synthesis of the enzyme. This mutation lies within a previously unidentified segment of highly conserved amino acid sequence homology that is shared among the largest subunits of eucaryotic nuclear RNA polymerases. Another temperature-sensitive mutation, *rpa190-2*, creates a UGA nonsense codon.

In eucaryotic cells a system of three distinct RNA polymerases has evolved for the expression of nuclear-encoded genes. These enzymes have been extensively studied in vitro; and it is well established that RNA polymerases I, II, and III (or A, B, and C) transcribe rRNA, mRNA, and tRNAs and 5S RNA, respectively (31). While these activities have distinct functional roles in the cell, they are closely related immunologically and in subunit composition (36). Each enzyme contains two large subunits, the larger of which is homologous to the β′ subunit of RNA polymerase from *Escherichia coli* and the smaller of which is homologous to the β subunit (1, 2, 5, 41). However, unlike the *E. coli* holoenzyme, which has the relatively simple $\sigma\alpha_2\beta\beta'$ subunit structure, the eucaryotic RNA polymerases have many more small subunits, none of which appear to be closely related to the α or σ subunits of *E. coli* RNA polymerase by immunological criteria (14). Some of the small subunits are shared among all of the three eucaryotic polymerases, while others are shared only between RNA polymerase I and III. Still others are unique for each enzyme.

Of the eucaryotic RNA polymerases, the yeast enzymes are among the best characterized (36). Most of our knowledge about these enzymes has been gained from in vitro studies, and these have been informative. However, it is often not clear how well the observed in vitro characteristics apply to the situation in growing cells, and one would like to complement these experiments with in vivo analyses. In particular, it would be advantageous to exploit the powerful yeast genetic techniques to gain insight into the manner in which these enzymes interact with their templates and transcription factors. Until recently, the genetic approach to the study of yeast RNA polymerases has been hampered by a lack of mutants. The recent cloning of many of the genes encoding subunits for the yeast enzymes (15, 30, 43) has allowed utilization of the powerful method of in vitro mutagenesis and in vivo screening for conditional mutants. Studies of this type have resulted in the successful isolation of temperature-sensitive mutants carrying alterations in the largest subunits of RNA polymerases II (3, 12, 24, 25) and III (R. Gudenus, S. Mariotte, A. Nonne, A. Ruet, S. Memet, A. Sentenac, and P. Thuriaux, submitted for publication) and the 40-kilodalton (kDa) subunit shared between RNA polymerases I and III (21). However, no mutants defective only in RNA polymerase I have been isolated in yeasts or in any other organism.

While RNA polymerases II and III utilize multiple DNA templates, RNA polymerase I is unique in that its sole known function is the transcription of the rRNA genes. In yeasts, the cellular concentration of polymerase I is controlled by the growth rate and its in vivo activity is responsive to a number of environmental signals (35, 37). Because of its key role in ribosome biosynthesis, regulated activity, and relative functional simplicity, we began a mutational analysis of this enzyme by mutagenizing the *RPA190* gene which encodes the largest subunit of yeast polymerase I. In

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TABLE 1. Yeast strains used in this study

Strain	Genotype
NOY80	*MATa ura3-52 leu2-3,112 can*^{r}
NOY205	*MATα ura3-52 leu2-3,112 his4-Δ401 trp1-Δ1*
NOY207	*MATa*/α diploid made by mating NOY80 × NOY205
NOY208	NOY207 transformed to Ura$^+$ phenotype with the linear 5.3-kb *Pvu*II-*Eco*RI fragment from pNOY3064; strain is heterozygous for the *rpa190Δ::URA3* null mutation
NOY210	*MATα ura3-52 leu2-3,112 trp1-Δ1 can*^{r}
NOY222	*MATα rpa190Δ::URA3 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r} pNOY20 (*LEU2*, *CAN*^{s}, *RPA190*) (NOY208 was transformed to Leu$^+$ with pNOY20 and subsequently sporulated to yield this haploid strain)
NOY244	*matα rpa190Δ::URA3 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r} pNOY16 (*TRP1*, *RPA190*) (constructed by transforming NOY222 with pNOY16 and subsequent curing of plasmid pNOY20)
NOY260[a]	*matα RPA190 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r}
NOY259[a]	*matα rpa190-1 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r}
NOY264[a]	*matα rpa190-2 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r}
NOY265[a]	*matα rpa190-3 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r}
NOY266[a]	*matα rpa190-4 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r}
NOY267[a]	*matα rpa190-5 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can*^{r}

[a] NOY260 was constructed by subjecting NOY244 to the 5-fluoroorotic acid selection as described in the text. The strains carrying the *rpa190* mutant alleles were constructed by subjecting strains similar to NOY244 (differing only in the *rpa190* allele carried on the pNOY16 derivative) to the 5-fluoroorotic acid selection. For example, NOY264 was derived from a strain carrying pNOY24 (Table 2).

this report we describe the isolation and characterization of five temperature-sensitive alleles. These mutations provide a first step in the genetic studies of ribosomal DNA transcription in yeasts.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. YEPD is 2% yeast extract, 1% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), and 2% glucose. Synthetic dextrose (glucose) medium (SD) was prepared as described previously (39). SD complete medium was SD medium supplemented with all of the base and amino acid additions described previously (39). Certain omissions were made for particular experiments, as designated.

Plasmids. The construction of the *rpa190Δ::URA3* null allele is described in the legend to Fig. 1. Construction of the plasmids used for the mutagenesis and screening procedures are described in the legend to Fig. 2. A complete listing of all the plasmids used in this study is given in Table 2. The set of plasmids used for the fine-structure mapping are described in Table 3.

In vitro mutagenesis with hydroxylamine. pNOY16 DNA was treated with hydroxylamine as described previously (32), except that the incubation at 37°C was for 24 h. In some experiments the mutagenized DNA was transformed into *E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and the plasmid DNA was amplified and subsequently used for yeast transformation. Alternatively, the mutagenized plasmid DNA was used directly for transformation into yeasts. Both procedures yielded temperature-sensitive mutants.

Screening yeast transformants for temperature sensitivity. A flow chart of the plasmid shuffle system is shown in Fig. 3. Hydroxylamine-mutagenized pNOY16 was used to transform NOY222 to a Trp$^+$ phenotype on SD complete medium plates (with all the base and amino acid supplements except tryptophan, leucine, and uracil) at 30°C by using the lithium acetate procedure (16). Transformants were then subjected to the following plasmid shuffle screening procedure. Individual Trp$^+$ transformants were patched out onto the same type of agar medium used for the transformation selection and grown for 2 days at 30°C. They were replica plated onto YEPD agar and grown for 1 to 2 days at 23°C on the nonselective medium, to allow for loss of pNOY20. Since pNOY20 is a very large 2μm-based plasmid, it was lost at a high frequency during growth on the nonselective medium. In contrast, the *CEN ARS* plasmid pNOY16 was relatively stable. These plates were then replicated onto minimal agar containing canavanine and leucine at 23°C to select for cells that had lost pNOY20. After 2 days most of the patches gave rise to confluent canavanine-resistant cells. At this point, the efficiency of the mutagenesis could be evaluated by scoring the number of isolates that failed to grow at 23°C. Typically, the frequency of mutants defective for *rpa190* at 23°C was 4 to 5%. Finally, the plates were replica plated to the same type of agar at 37°C to screen for a temperature-sensitive phenotype. Plasmid DNA was recovered in *E. coli* DH5α (Bethesda Research Laboratories) from isolates that appeared to be temperature sensitive and was retransformed into NOY222. The resulting yeast transformants were subjected to the same replica-plating scheme as described above, and five plasmids that reconfirmed a temperature-sensitive phenotype were selected for further analysis. The five temperature-sensitive alleles generated in this way are listed as *rpa190-1* through *rpa190-5*.

Transfer of the mutant *rpa190* alleles to the chromosome. The five strains derived from the hydroxylamine mutagenesis (in which a mutant *rpa190* gene on a *CEN3 TRP1 ARS1* plasmid complemented the chromosomal *rpa190Δ::URA3* null allele) were streaked onto YEPD agar and allowed to grow at 23°C to allow for recombination or gene conversion between the plasmid *rpa190* allele and the chromosomal null mutation. A strain harboring unmutagenized pNOY16 was included as a control. These plates were then replica-plated onto agar containing 5-fluoroorotic acid (6) at 23°C, and Ura$^-$ colonies were selected. These were screened for the loss of the *TRP1 ARS1/CEN3* plasmid by checking for a Trp$^-$ phenotype (typically, about 10% of the Ura$^-$ colonies were also Trp$^-$). All of the Ura$^-$ isolates derived from strains originally containing mutant plasmids were temperature sensitive for growth at 37°C (Ts$^-$), while the isolate derived from the strain that contained unmutagenized pNOY16 was Ts$^+$. Restoration of the wild-type chromosomal configuration at the *RPA190* locus and loss of the

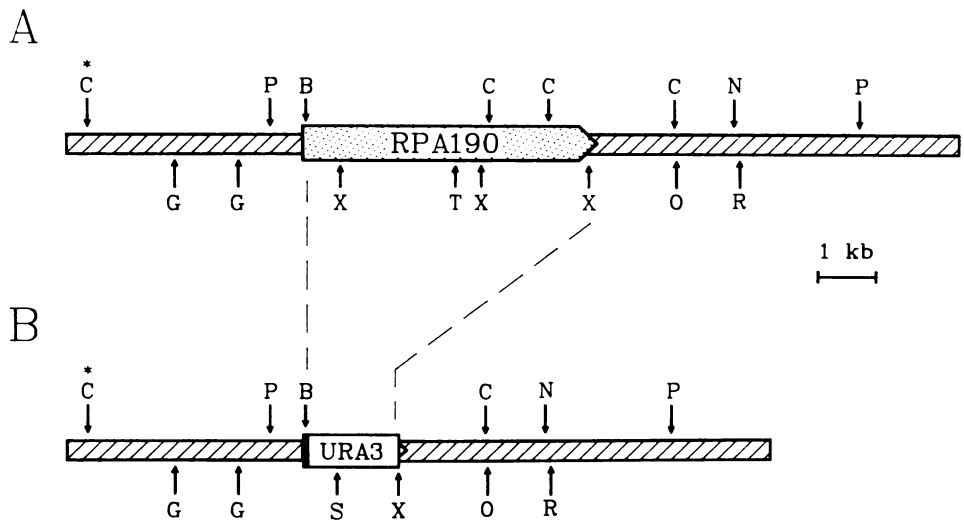

FIG. 1. The *RPA190* gene and construction of the null allele. (A) Diagram of the chromosomal segment of yeast DNA containing the *RPA190* gene (see reference 22 for the DNA sequence). Restriction enzyme site designations are as follows: B, *Bam*HI; C, *Cla*I; R, *Eco*RI; G, *Bgl*II; N, *Nhe*I; O, *Xho*I; P, *Pvu*II; S, *Sma*I; T, *Pst*I; X, *Xba*I. For the *Xba*I and *Pst*I sites, only those sites within the structural gene are shown. (B) Construction of the null allele *rpa190Δ::URA3*. Starting with the 1.1-kb *URA3 Hin*dIII fragment cloned into the *Hin*dIII site of pBR322, an *Xba*I linker was ligated to the *Eco*RI site which was blunt-ended with the action of the Klenow fragment of DNA polymerase. The 1.4-kb *Xba*I-*Bam*HI fragment containing the *URA3* gene was ligated into the plasmid pA3 (22), which was cut with *Bam*HI and then partially cut with *Xba*I. The resulting plasmid, pNOY3064, had the *URA3* segment replacing almost the entire coding sequence of *RPA190*. Of the three *Xba*I sites within the *RPA190* structural gene, the site closest to the 3′ end of the gene is the location of the fusion to the *URA3* segment (this *Xba*I site is numbered 4875 in the published *RPA190* sequence [22]). A strain carrying this null allele at the chromosomal *RPA190* locus was constructed by using the one-step gene transplacement procedure (33). This was done by transforming the diploid NOY207 with the 5.3-kb *Pvu*II-*Eco*RI DNA fragment from pNOY3064 containing the *URA3* gene and the *RPA190*-flanking sequences. The resulting Ura^+ strain, NOY208, was sporulated, and only two viable spores from each tetrad were recovered; each of these was phenotypically Ura^-. Strain NOY222 was constructed by transforming NOY208 with pNOY20 (Fig. 2B) followed by sporulation. This haploid strain (NOY222) contained the *rpa190Δ::URA3* null allele on the chromosome complemented by the *RPA190* gene on the plasmid.

plasmid and *rpa190Δ::URA3* null alleles for each Ts^- strain and the wild-type control was confirmed by Southern analysis (Fig. 4).

Fine-structure mapping and sequencing of the mutant alleles. Fine-structure mapping was accomplished by transforming the strains carrying the mutant alleles on the chromosome (strains NOY259 and NOY264 through NOY267) to a Ura^+ phenotype with the set of plasmids containing portions of the wild-type *RPA190* gene in a *URA3*/2μm-based vector (see Table 3 and Fig. 5). A marker rescue experiment was performed by allowing the transformants to grow in SD liquid medium supplemented with histidine, leucine, and tryptophan at 23°C until the late log phase and then were shifted to 37°C overnight. The cells were collected by centrifugation, and about 10^8 cells were spread onto SD complete agar medium with all supplements except uracil. After 2 days of incubation at 37°C, the appearance of Ts^+ colonies was scored. Alleles *rpa190-1* and *rpa190-5* were mapped to a 630-base-pair (bp) *Bam*HI-*Xba*I fragment, and alleles *rpa190-2* and *rpa190-3* were mapped to a 675-bp *Nsi*I-*Pst*I fragment. These fragments were subcloned from the corresponding mutant pNOY16 derivatives into M13mp18 and M13mp19 vectors (26), and sequencing was carried out by the dideoxy method (34). In each case, the whole fragment to which the mutation was mapped was sequenced.

RNA accumulation measurements. Strains were grown at 23°C for several generations in SD complete liquid medium supplemented with all the amino acids and bases (except methionine and lysine) and containing [^{3}H]uridine (40 μg/ml, 5 μCi/ml). When the optical density at 600 nm of the cultures reached approximately 0.05, one-half of the culture was

TABLE 2. Plasmids used in this study

Plasmid	Description
pA3	17 kb of yeast DNA containing *RPA190* in YRp7 (22)
pNOY3064	Most of the *RPA190*-coding region of pA3 replaced with *URA3* gene; the 1.1-kb *URA3* fragment replaces *RPA190* DNA from nucleotides 50 (*Bam*HI) to 4875 (*Xba*I) (see published sequence [30]); the 5.3-kb *Pvu*II-*Eco*RI fragment from this plasmid was used to construct the *rpa190Δ::URA3* allele in strain NOY208
pNOY16	*TRP1 ARS1, CEN3 RPA190* (Fig. 2A)
pNOY20	*LEU2, CAN*^{s}, 2μm, *RPA190* (Fig. 2B)
pNOY23	*rpa190-1* allele generated by treating pNOY16 with hydroxylamine
pNOY24	*rpa190-2* allele generated by treating pNOY16 with hydroxylamine
pNOY34	*rpa190-3* allele generated by treating pNOY16 with hydroxylamine
pNOY35	*rpa190-4* allele generated by treating pNOY16 with hydroxylamine
pNOY36	*rpa190-5* allele generated by treating pNOY16 with hydroxylamine

shifted to 37°C and the other was kept at 23°C. Accumulation of the ^3H label was followed periodically by removing 1 ml of the culture and placing it into 1 ml of 10% trichloroacetic acid and 5 mg of uridine per ml on ice. Acid-precipitable ^3H counts per minute were then determined.

Pulse-labeling of cellular RNA and preparation of RNA for hybridization. Strains NOY260 (*RPA190*), NOY259 (*rpa190-1*), and NOY265 (*rpa190-3*) were transformed to Ura$^+$ with plasmid YCp50 (a *URA3 CEN4 ARS1* yeast-*E. coli* shuttle vector [27]), and the resulting strains were grown in SD complete medium (except methionine, lysine, and uridine) at 23°C. Portions of the cultures were shifted to 37°C for 6.5 h prior to labeling. Cultures of 5 ml were labeled with [^3H]uridine (195 mCi/mg, 250 μCi/ml) for 10 min, and the labeling was stopped by the addition of 10 ml of ice-cold water and cooling on ice. Cells were collected by centrifugation at 2°C, washed with cold water, frozen quickly, and stored at −70°C. The frozen cell pellet was suspended in 100 μl of LET buffer (0.1 M LiCl, 1 mM EDTA, 0.1 M Tris hydrochloride [pH 7.8]), and glass beads (diameters, 0.45 to 0.50 mm) were added to the meniscus. The cells were broken by shaking with a vortex mixer. Sodium dodecyl sulfate (4 μl of 10%) was added along with 300 μl of LET buffer and 400 μl of LET-saturated phenol-chloroform-isoamyl alcohol (25:20:1). After three extractions, the RNA was precipitated twice with ethanol and suspended in hybridization solution (0.1 M Tris hydrochloride [pH 8.0], 0.6 M NaCl, 0.02 M EDTA, 0.1% sodium dodecyl sulfate, 0.02% polyvinylpyrrolidone, 50% [vol/vol] formamide, 100 μg of *E. coli* tRNA per ml).

RNA-DNA hybridization. Purified single-stranded M13NOY4, M13NOY10, or M13mp8 DNA (25 μg) was immobilized on a filter (pore size, 0.45 μm; diameter, 25 mm; Millipore Corp., Bedford, Mass.); and the filters were baked for 3 h at 80°C under vacuum. M13NOY4 contains the 2.7-kilobase (kb) *Eco*RI A fragment of *Saccharomyces cerevisiae* ribosomal DNA (28) ligated into the *Eco*RI site of M13mp18. M13NOY10 contains the 1.2-kb *Sph*I-*Hin*dIII fragment of *S. cerevisiae* ribosomal DNA containing the 5S RNA gene ligated into M13mp19. The orientation of the cloned fragments for both probes is such that the single-stranded DNA hybridizes 25S rRNA or 5S RNA. For hybridization, each glass vial contained 1.5 ml of hybridization solution, including [^3H]RNA, one M13NOY4 or M13NOY10 filter, and one M13mp8 filter. Hybridization was carried out at 41°C for 16 h. The filters were washed and treated with RNase A, and the radioactivities were determined as described before (18). A linear relationship was observed between [^3H]RNA input to the hybridization and ^3H counts per minute recovered on the filters for each sample, establishing that the DNA probe was in excess.

In vitro RNA polymerase I activity. One-liter cultures were grown in SD medium at 23°C or first grown at 23°C and then shifted to 37°C for 8 h prior to harvesting. Cells were collected and disrupted, and RNA polymerase I was partially purified by using a microscale RNA polymerase I preparation (29) through the phosphocellulose step. The 0.4 M eluate fraction from the phosphocellulose step, which has no RNA polymerase II or III activity (21), was concentrated; and protein concentrations were measured with a protein microassay (Bio-Rad Laboratories, Richmond, Calif.). The fractions were then diluted with the same buffer that was used for elution [20 mM Tris hydrochloride (pH 8.0), 10 mM 2-mercaptoethanol, 0.5 mM sodium EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 M $(NH_4)_2SO_4$], so that the protein concentration of each was equal. Frac-

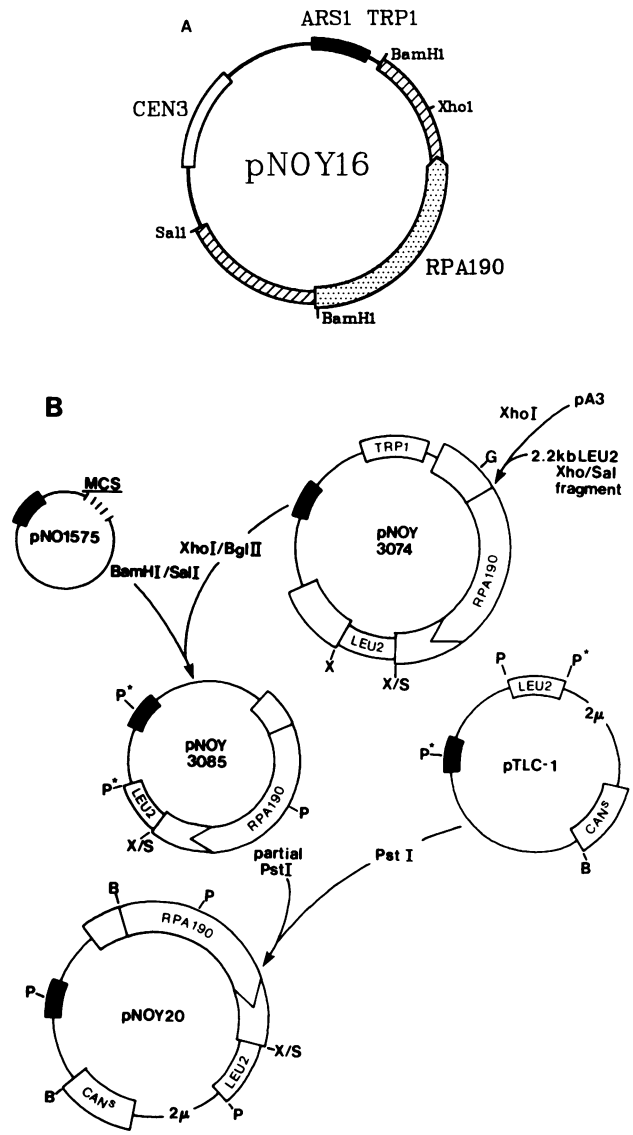

FIG. 2. Plasmids used in the plasmid shuffle scheme. (A) Description of pNOY16 (the mutagenesis plasmid). The *Cla*I site marked with an asterisk in Fig. 1A was converted to a *Sal*I site with the use of a linker (TAB linker pCGTCGA; Pharmacia Fine Chemicals, Piscataway, N.J.). Plasmid pNOY16 (19.5 kb) was then constructed by inserting the 11-kb *Sal*I(*Cla*I)-*Nhe*I (which contains *RPA190*; Fig. 1A) fragment between the *Sal*I-*Bam*HI sites of the *TRP1 ARS1/CEN3* vector pTC3 (38). The *Nhe*I (fragment)-*Bam*HI (vector) ligation was illegitimate, resulting in retention of the *Bam*HI site at this junction. (B) Construction of pNOY20 (the resident plasmid). The 2.2-kb *Xho*I-*Sal*I fragment containing the *LEU2* gene was ligated into the *Xho*I site of pA3 (22) to form pNOY3074. The 10.2-kb *Xho*I-*Bgl*II fragment from pNOY3074 was ligated into the vector pNO1575 (17) at the *Sal*I-*Bam*HI sites, resulting in pNOY3085. This plasmid was digested partially with *Pst*I, the 11-kb fragment containing the *RPA190* and *LEU2* genes was isolated and ligated with the 10.2-kb *Pst* fragment from pTLC-1 containing the *CAN1* gene and the 2μm replicator sequence (7) to yield pNOY20. The *Pst*I sites in pTLC-1 and pNOY3085 that were ligated to form pNOY20 are indicated with asterisks. Abbreviations: P, *Pst*I; B, *Bam*HI; MCS, multiple cloning site (derived from pUC9); G, *Bgl*II; X/S, result of ligating an *Xho*I and a *Sal*I site together. Filled boxes indicate the location of the β-lactamase gene.

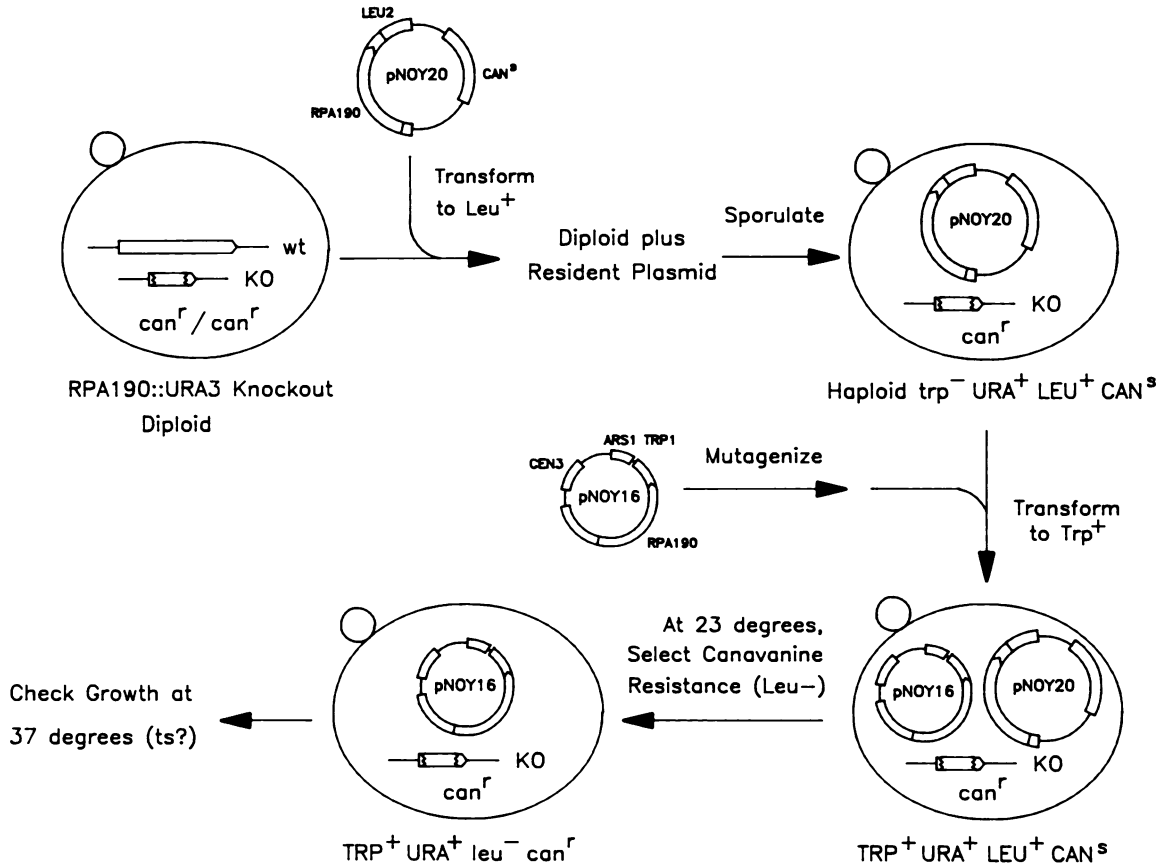

FIG. 3. Flow diagram of the plasmid shuffle scheme to isolate mutants (see the text). wt, Wild type.

tions (usually 5 μl) were used to assay RNA polymerase activity in a total volume of 75 μl. In addition to the RNA polymerase I fraction, a typical reaction mixture contained 50 mM Tris hydrochloride (pH 7.4), 2 mM $MnCl_2$, 0.6 mM GTP, 0.6 mM CTP, 0.6 mM ATP, 6 mM NaF, 0.1 mM UTP containing 3 μCi of [^{3}H]UTP or [^{32}P]UTP, 50 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, 50 μg of denatured salmon sperm DNA per ml, and 50 μg of native salmon sperm DNA per ml. The reaction mixtures were incubated at 23°C for 20 min. The reactions were stopped by transferring 70 μl into 2 ml of 5% trichloroacetic acid–1% NaH_2PO_4 on ice. The acid-precipitable material was collected on glass fiber filters (GF/C; Whatman, Inc., Clifton, N.J.), and the amount of radioactivity was determined by scintillation counting.

RESULTS

In vitro mutagenesis and screening for temperature-sensitive *RPA190* mutant. Hydroxylamine was used to mutagenize the *S. cerevisiae RPA190* gene in vitro, and a plasmid shuffle system was implemented in order to screen efficiently for temperature-sensitive mutations. This type of system has been used to mutagenize other essential yeast genes (9, 21). A diagram of the plasmids that were used is shown in Fig. 2. A flow diagram of the plasmid shuffle system is shown in Fig. 3. The cell carries the null allele *rpa190Δ::URA3* (Fig. 1) at the *RPA190* locus on chromosome XV (the *RPA190* gene has been mapped to chromosome XV [22]; M. Wittekind, Ph.D. thesis, University of Wisconsin, Madison, 1988). This is complemented by a plasmid carrying a wild-type version of the gene. This plasmid, pNOY20, is called the resident plasmid. A second plasmid, pNOY16, called the mutagenesis plasmid, also carries a wild-type version of the gene. This plasmid was mutagenized in vitro and transformed into the strain carrying the resident plasmid.

In this particular version of the scheme, the resident plasmid also carried the wild-type *CAN1* gene (7, 42). We selected against pNOY20 by spreading the double transformants onto agar containing canavanine and leucine, because the chromosomal allele is *can*^{r}, which is recessive to *CAN*^{S} (leucine is needed because the cells are Leu$^-$ in the absence of the pNOY20-borne *LEU2* gene). With the wild-type copy of the *RPA190* gene from pNOY20 absent, the only source of the large subunit of RNA polymerase I was the mutagenized pNOY16 derivative. The canavanine selection was done at the permissive temperature (23°C) to allow growth of temperature-sensitive mutants. The colonies were then screened for a temperature-sensitive phenotype at 37°C. Since the mutant alleles were on the plasmid, they could be easily recovered for analysis.

The level of hydroxylamine mutagenesis used was such that the frequency with which strains defective for *rpa190* at 23°C were generated was 4 to 5%. Of approximately 15,000 colonies, seven were temperature sensitive for growth at 37°C. The plasmids were recovered from the seven strains, and on reintroduction into yeasts, five were found to confer a temperature-sensitive phenotype when they were used to complement the *rpa190Δ::URA3* null allele.

Transfer of mutant alleles to the chromosomal *RPA190* locus. The five mutations and the wild-type allele were transferred to the chromosome by a variation of the one-step

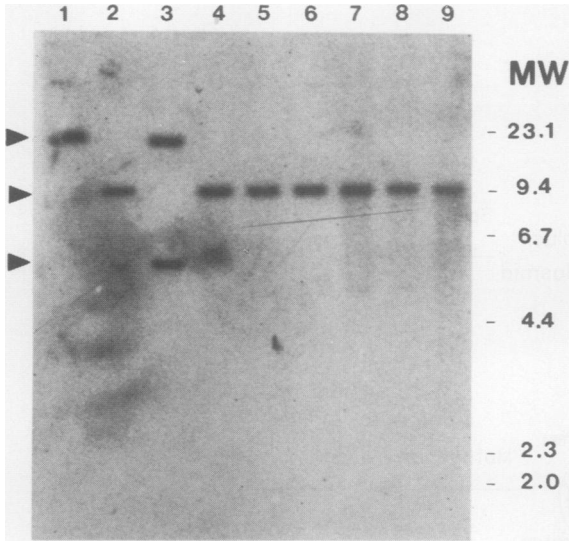

FIG. 4. DNA-blot gel analysis of *Pvu*II-digested DNA to confirm the transfer of mutant *rpa190* alleles to the chromosomal *RPA190* locus. Chromosomal DNA was isolated, cut with *Pvu*II, and separated on a 0.7% agarose gel. After transferring to nitrocellulose, the DNA was probed with the 3.8-kb *Pst*I-*Xho*I fragment specific for the 3′ end of the *RPA190* gene and the 3′-flanking sequences. An autoradiogram is shown. The arrowheads on the left indicate the position of the 19.5-kb linearized pNOY16 plasmid (top arrowhead) (there is only a single *Pvu*II site in pNOY16), the approximately 10.5-kb wild-type *RPA190 Pvu*II fragment (middle arrowhead), and the 5.3-kb *Pvu*II fragment from the *rpa190Δ::URA3* allele (bottom arrowhead). Lane 1, plasmid pNOY16 DNA linearized with *Sal*I; lane 2, strain NOY210 (wild-type *RPA190*); lane 3, strain NOY244 (*rpa190Δ::URA3* on chromosome complemented by pNOY16); lanes 4 through 9, strains NOY260 (*RPA190*), NOY259 (*rpa190-1*), NOY264 (*rpa190-2*), NOY265 (*rpa190-3*), NOY266 (*rpa190-4*), NOY267 (*rpa190-5*), respectively. Molecular weight (MW) markers (in thousands) are bacteriophage lambda DNA cut with *Hin*dIII.

gene replacement procedure (33), generating six isogenic strains that differed only at the allele present at the chromosomal *RPA190* locus (see above). The integrity of the replacements was proven by Southern analysis (Fig. 4). This set of strains was used in all subsequent mapping and physiology experiments.

Fine-structure mapping and sequencing. Marker rescue was used for fine-structure mapping of the *rpa190* mutations. The mapping strategy is depicted in Fig. 5, and the results are given in Table 3. Alleles *rpa190-1* and *rpa190-5* were mapped to a 630-bp *Bam*HI-*Xba*I fragment near the 5′ end of the gene. This segment spans region I, a region of homology shared among all of the eucaryotic RNA polymerase large-subunit genes (2, 22) (Fig. 5). Alleles *rpa190-2* and *rpa190-3* mapped to a 675-bp *Nsi*I-*Pst*I fragment, which falls between conserved regions III and IV. The strain containing allele *rpa190-4*, although it was temperature sensitive, proved to be too leaky to score by this procedure, and so it remained unmapped.

DNA sequence analysis of these fragments has, in fact, revealed mutational alterations. These alterations and the deduced amino acid changes are shown in Fig. 6. Figure 6 also shows the amino acid sequence homology to other RNA polymerase large subunits. All mutations were GC to AT transitions, consistent with the mutational specificity of hydroxylamine (40). Alleles *rpa190-1* and *rpa190-5* are tightly clustered within a highly conserved portion of region I, which has been suggested to be involved in the binding of zinc (22). Both mutations change amino acids that are invariant among all of the large subunits of eucaryotic RNA polymerases that have been sequenced to date. The *rpa190-5* mutation is a His to Tyr substitution at position 75 of the wild-type protein sequence. This changes one of the histidines that make up the consensus sequence of the putative zinc-binding Cys-X_2-Cys-X_9-His-X_2-His motif (Fig. 6). The *rpa190-1* allele substituted an aspartate for the conserved glycine residue at position 77, which lies between the two histidines in the consensus sequence. These mutations were very similar phenotypically (see below).

The *rpa190-3* allele contained a glycine to aspartate missense mutation at position 728 in the amino acid sequence of *RPA190*. This mutation fell within a short amino acid sequence which, we found, was very highly conserved among all three of the yeast RNA polymerase largest subunits and the mouse RNA polymerase II largest subunit (Fig. 6). The glycine residue that was altered in *rpa190-3* was conserved among all of these sequences. This stretch of homology fell between the previously identified regions III and IV that are conserved in the sequences of the largest subunits of the eucaryotic RNA polymerases (8). We have been unable to find significant homology when trying to align this region with the amino acid sequence of the β′ subunit of *E. coli* RNA polymerase or with the 147-kDa RNA polymerase sequence from vaccinia virus (8).

The *rpa190-2* mutation coded for a UGA stop codon at amino acid position 679. This was unexpected since the strain is not known to carry any UGA suppressors. Truncation of the subunit polypeptide at this point in the coding sequence would remove almost 60% of the amino acid residues. This would remove many sequences which are highly conserved among the other RNA polymerase largest subunits. The two entire DNA fragments from the *rpa190-4* mutant plasmid corresponding to the two fragments in which the other four alleles were mapped were also sequenced, and no mutational alteration was found. So, while the *rpa190-4* mutation remains unmapped, it must be located in a different region than the other mutations.

Physiological and biochemical characterization of the *rpa190* mutants. The growth rates of the mutants were determined at the permissive and nonpermissive temperatures (Fig. 7). Strains carrying alleles *rpa190-1*, *-2*, or *-5* grew at approximately 75% of the wild-type rate at 23°C; and the kinetics of growth inhibition at 37°C were very similar for these three mutant strains. Those carrying allele *rpa190-3* or *rpa190-4* grew almost at the wild-type rate at 23°C. At 37°C, the two strains behaved quite differently. With the *rpa190-4* strain, inhibition was only slight and the cells continued to

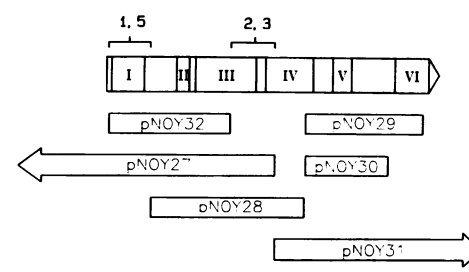

FIG. 5. DNA fragments used for marker rescue experiments to map the mutant *rpa190* alleles. The segments I through VI within the *RPA190* coding region are the conserved regions (8), as discussed in the text. The regions where the mutations were localized are indicated (for mutant alleles 1, 2, 3, and 5) (for further details, see Table 3 and the text).

TABLE 3. Fine-structure mapping

Plasmid[a]	Plasmid insert			Marker rescue results for the following *rpa190* allele no.[c]:				
	Ends	Size (bp)	Spanning nucleotides[b]	-1	-2	-3	-4	-5
pNOY27	*Bgl*II-*Pst*I	3,000	(5')–2575	+++	+++	+	ND	+++
pNOY28	*Xba*I-*Xba*I	2,355	680–3035	−	+++	++	ND	−
pNOY29	*Xba*I-*Xba*I	1,850	3035–4875	−	−	−	ND	−
pNOY30	*Sau*3A-*Sau*3A	1,290	3040–4330	−	−	−	ND	−
pNOY31	*Pst*I-*Xho*I	3,900	2575–(3')	−	−	−	ND	−
pNOY32	*Bam*HI-*Nsi*I	1,900	50–1900	+	−	−	ND	+
YEp352		0		−	−	−	ND	−

[a] All plasmids are derivatives of the 2μm-*URA3* shuttle vector Yep352 (11). The *RPA190* DNA fragments were cloned into the multiple cloning sites of the vector.

[b] *RPA190* numbering system taken from the published sequence (22). The first nucleotide of the ATG initiator codon is numbered 1, and the G of the TAG terminator codon is numbered 4998. (5') and (3') refer to the DNA flanking the *RPA190* structural gene on the 5' and 3' ends, respectively.

[c] Marker rescue was carried out as described in the text. For each transformant, analysis was done in duplicate, and consistent results were obtained in all cases. The number of Ts^+ colonies scored for about 10^8 cells spread were +++, >50 colonies per plate; ++, 10 to 50 colonies per plate; +, 2 to 10 colonies per plate; −, no colonies detected; ND, not determined.

grow very slowly for a long time. The *rpa190-3* strain showed a tighter growth inhibition at the nonpermissive temperature. On plates, it was clear that growth at 37°C was inhibited much more in the *rpa190-3* strain than in the *rpa190-4* strain.

The RNA accumulation rates of the mutant strains were examined at the permissive temperatures and after a shift to the nonpermissive temperature (Fig. 8). All of the mutants had defects in stable RNA accumulation at the nonpermissive temperature. The RNA accumulation rates matched the growth rates at 23°C, and while all of the mutants appeared to continue to accumulate RNA at 37°C, the rates were no longer exponential. These data are consistent with the conclusion that the mutations are in the gene coding for the

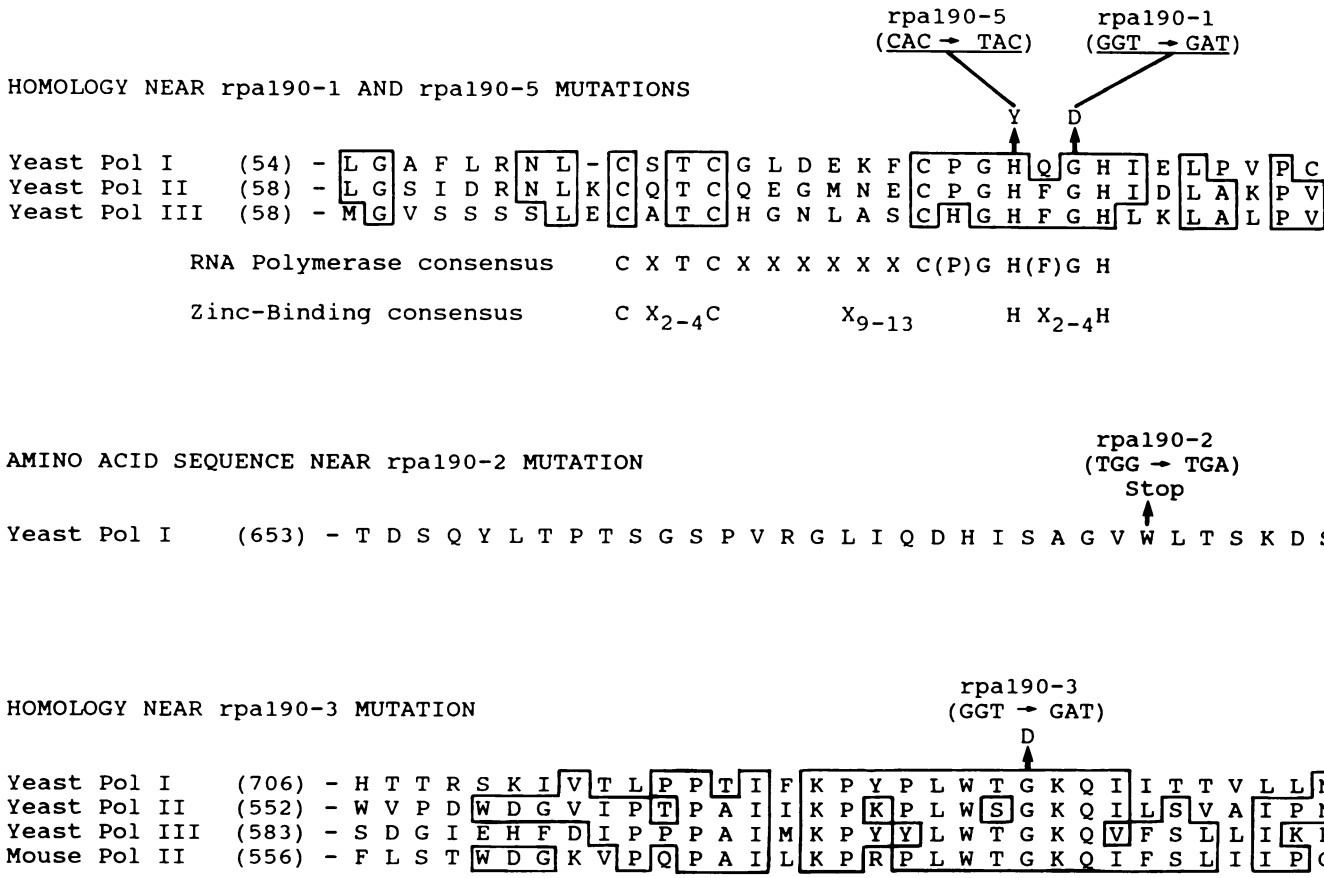

FIG. 6. Sequence alterations found in the *rpa190* mutations and amino acid sequences of homologous regions from other RNA polymerase large subunits. A segment of the amino acid sequence near the sites of the various *rpa190* mutational alterations is shown along with the homologous regions from other RNA polymerase large subunits. A portion of the region I homology consensus for the RNA polymerase large subunits and a proposed zinc-binding site consensus sequence (4) is shown below the *rpa190-1* and *rpa190-5* mutations. The RNA polymerase largest subunit sequences from yeast polymerase I (22), mouse polymerase II (1), and yeast polymerases II and III (2) are shown. The numbers in parentheses near the beginning of each sequence indicate the position in the sequence of the first amino acid shown.

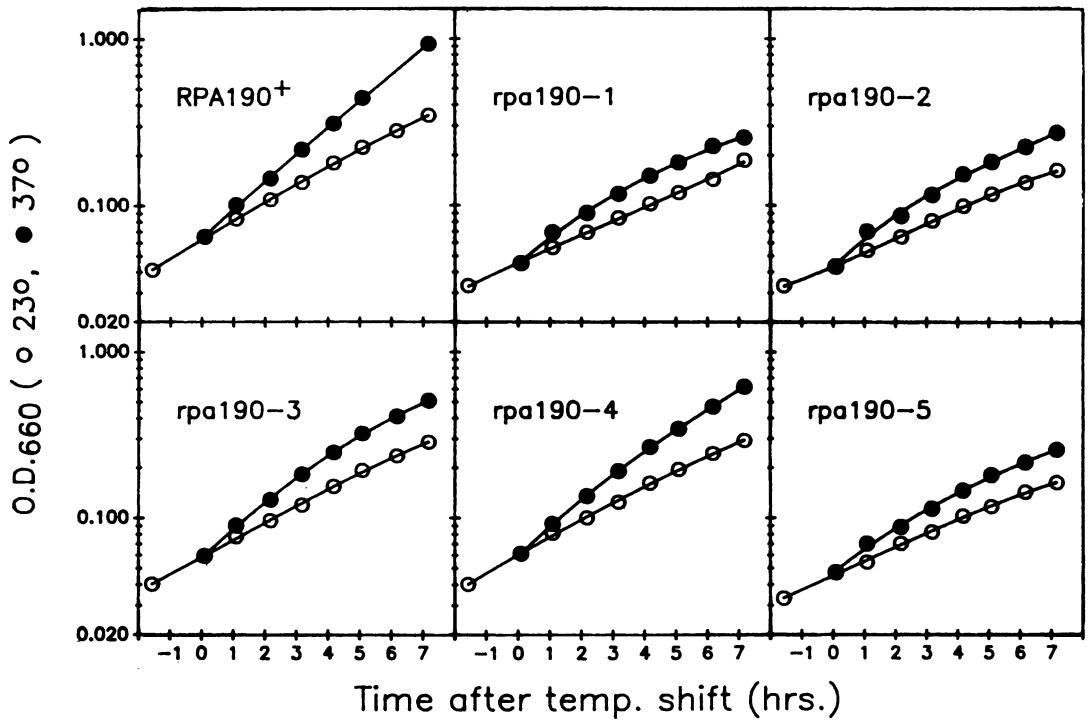

FIG. 7. Growth rates of the temperature-sensitive mutants. The wild-type strain NOY260 and the five isogenic temperature-sensitive strains NOY259 (*rpa190-1*), NOY264 (*rpa190-2*), NOY265 (*rpa190-3*), NOY266 (*rpa190-4*), and NOY267 (*rpa190-5*) were grown in SD complete medium (without methionine or lysine). The cultures were grown at 23°C and divided into two portions, and one of them was shifted to 37°C. Their growth rates were followed by reading the optical density at 660 nm (OD_{600}).

RNA polymerase I subunit A190. However, none of the mutations caused a quick shutoff of stable RNA accumulation. Therefore, it is possible that these mutations may cause defects in the synthesis of new RNA polymerase I, without affecting preexisting polymerase I, at the nonpermissive temperature.

The conclusion that the mutations specifically affect RNA polymerase I was further supported by analyzing pulse-labeled RNA synthesized in the strains each carrying *rpa190-1* or *rpa190-3* mutations by the RNA-DNA hybridization method. Cells were labeled with [^{3}H]uridine for 10 min at 23°C and at 6.5 h after the temperature shift to 37°C.

TABLE 4. Synthesis of rRNA and 5S RNA during a 10-min pulse in the *rpa190-1* and *rpa190-3* mutant strains[a]

RPA190 allele	Temp (°C)	Input RNA (cpm)	Radioactive RNA hybridized to probes for:			
			rRNA		5S RNA	
			cpm observed	% of total	cpm observed	% of total
RPA190	23	162,000	25,800	15.9 (1.0)	1,400	0.86 (1.0)
	37	52,800	7,640	14.5 (1.0)	429	0.81 (1.0)
rpa190-1	23	87,100	9,120	10.5 (0.66)	738	0.85 (0.99)
	37	47,200	1,260	2.7 (0.19)	570	1.21 (1.49)
rpa190-3	23	143,000	20,900	14.6 (0.92)	1,340	0.94 (1.09)
	37	30,000	1,030	3.4 (0.23)	402	1.34 (1.65)

[a] RNA-DNA hybridization was carried out as described in the text. The 37°C samples were from cells labeled at 6.5 h after the shift from 23 to 37°C. Three different amounts of [^{3}H]RNA were analyzed in separate vials for each sample. The value listed as input is the highest input. The value listed as RNA hybridized is an average of the [^{3}H]RNA recovered in hybrids for the different input [^{3}H]RNAs and is normalized to the highest amount of input [^{3}H]RNA. The ^{3}H counts per minute recovered ranged from 7 to 100 times the background for the rRNA probe and from 4 to 7 times the background for the 5S RNA probe. The numbers in parentheses are the percentages of total input [^{3}H]RNA normalized to the wild-type control. The 23 and 37°C values for the mutants are normalized to the 23 and 37°C values for the wild type, respectively. These values can be used to estimate the effects of mutations on the synthesis of rRNA and 5S RNA. An example for *rpa190-3* is given as follows. Assuming that the rRNA synthesized during a 10-min pulse represents about 60% of total RNA (unpublished data; see also Kim and Warner [19]) and the synthesis of mRNA and tRNA is not affected by the mutations, rRNA synthesized in the *rpa190-3* strain (x) relative to that in the wild type (1.0) at 37°C can be calculated from the following equation: $[x/(x + 0.4)]/(0.6/1.0) = 0.23$. Thus, $x = 0.064$, or 11% [(0.064/0.6) × 100] of the synthesis in the wild type. Similarly, the ratio (y'/y) of 5S RNA synthesized in the mutant *rpa190-3* (y') to that in the wild type (y) at 37°C can be calculated from the following equation: $[y'/(0.4 + 0.064)]/(y/1.0) = 1.65$. Thus, $y'/y = 0.77$; that is, the synthesis of 5S RNA in the mutant at 37°C was decreased by only a small amount (23%). It should be noted that this value (23%) was estimated indirectly by calculation and may not be accurate. In addition, it is possible that this slight inhibition, even if it is real, may not be transcriptional and may actually reflect decreased stability of the 5S RNA transcript during inhibition of ribosome assembly because of limiting transcription of rRNA genes.

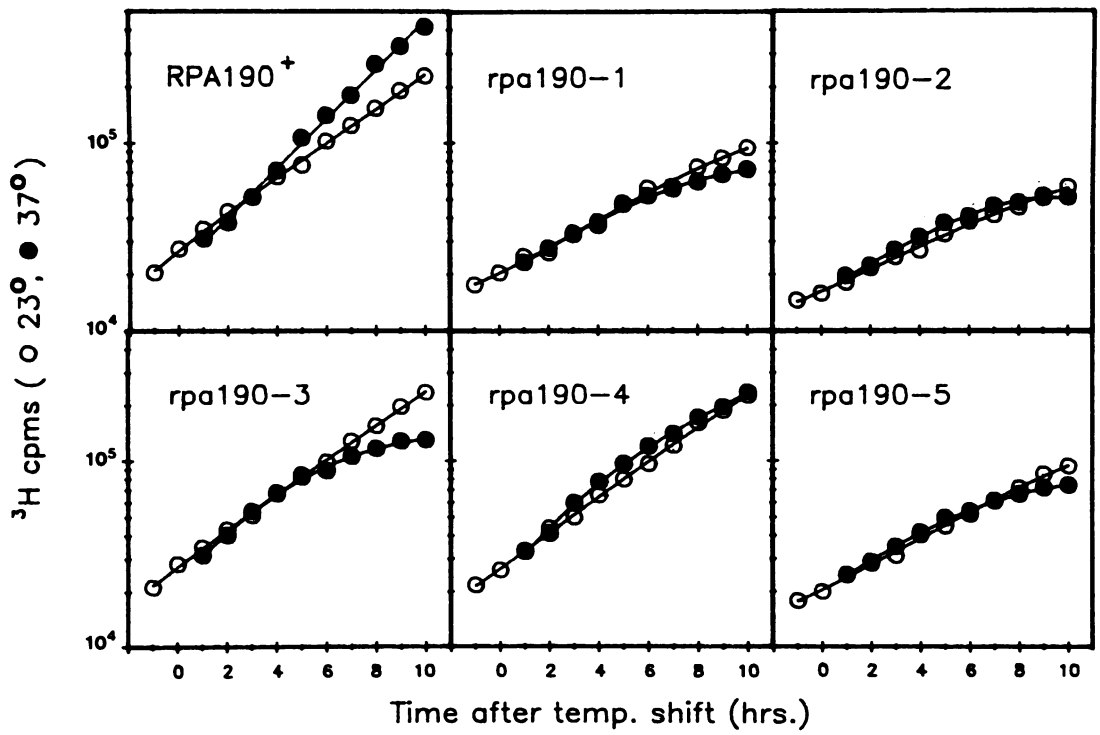

FIG. 8. RNA accumulation rates of the temperature-sensitive mutants. The wild-type control strain NOY260 and the temperature-sensitive strains NOY259 (*rpa190-1*), NOY264 (*rpa190-2*), NOY265 (*rpa190-3*), NOY266 (*rpa190-4*), and NOY267 (*rpa190-5*) were labeled; and the accumulation of RNA was followed as described in the text.

RNA was isolated, and the amounts of radioactive rRNA as well as 5S RNA were determined by using suitable probes. Under the nonpermissive condition used, differential synthesis rates of rRNA in the *rpa190-1* and *rpa190-3* strains were only about 20% that of the control *RPA190* strain, whereas differential synthesis rates of 5S RNA in the mutants was about 50 to 65% higher than that in the control strain (Table 4). Thus, it is evident that the mutations preferentially affected rRNA synthesis. The synthesis of 5S RNA, which is catalyzed by RNA polymerase III, was affected much less (see footnote *a* to Table 4 for a quantitative consideration).

We examined RNA polymerase activity in partially purified extracts made from the wild-type strain and the *rpa190-1* and *rpa190-3* mutant strains. The extracts were made from cells that were grown at 23°C or from cells that were shifted to 37°C for 8 h. A simple fractionation method was used to remove virtually all the polymerase II and III activities from the extracts. It can be seen from the results shown in Table 5 that both of the extracts from mutant cells grown at 23°C had polymerase I activity that was decreased by approximately 35% relative to that of the wild type. However, the extracts made from mutant cells that were shifted to 37°C for 8 h had polymerase I specific activities that were reduced by about 80% relative to that of the wild type. These results are consistent with the in vivo results described above (see footnote *a* to Table 4). Results of preliminary experiments indicate that the polymerase I activities of the extracts made from the two mutant strains grown at 23°C became totally inactivated by preincubation at 50°C for 10 min before the assay was done at 23°C. Preincubation reduced the wild-type extract polymerase I specific activity by only 20%. These results indicate that the polymerase I activities in these mutant strains are decreased by growing the cells at 37°C and that the polymerase I enzymes produced at 23°C are not as thermotolerant as the wild-type polymerase I.

DISCUSSION

Using a plasmid shuffle method, we isolated several temperature-sensitive mutants that specifically affected RNA polymerase I. Among the five mutants characterized, two (*rpa190-1* and *rpa190-5*) were in a putative zinc-binding region, which is found within conserved region I, as defined

TABLE 5. In vitro RNA polymerase I activities

Source[a]	cpm[b]	Normalized values	37°C/23°C[c]
Wild type			
NOY260 (23°C)	42,762	1.00	
NOY260 (37°C)	39,759	0.93	0.93 (1.00)
rpa190-1			
NOY259 (23°C)	27,019	0.63	
NOY259 (37°C)	5,473	0.13	0.21 (0.22)
rpa190-3			
NOY265 (23°C)	28,303	0.66	
NOY265 (37°C)	4,665	0.11	0.17 (0.18)

[a] Extracts were made from the indicated strains grown at 23°C or shifted from 23 to 37°C for 8 h prior to harvesting.

[b] RNA polymerase activities were measured at 23°C for cell extracts that were partially purified by phosphocellulose chromotography (see text). All assays were done in triplicate, and the average value is shown after subtraction of the background obtained in the absence of extracts (2,700 cpm).

[c] Values in parentheses are the percentages of the 37°C/23°C ratio to the normalized value (0.93) for the wild type.

by Broyles and Moss (8). Two previously isolated mutations in the yeast RNA polymerase II largest subunit (12) were also located close to these two *rpa190* mutations in this conserved region I. This suggests that this portion of the subunit is particularly sensitive to amino acid substitutions. Since only temperature-sensitive mutations were analyzed, it is not known how many of the null alleles fell within this region. The phenotypes of the *rpa190-1* and *rpa190-5* alleles are very similar in growth rate and RNA accumulation at the nonpermissive temperature, and it is likely that they perturb the region in similar ways. The region affected by these mutations is implicated in zinc-binding solely on the basis of its very high similarity to the zinc-binding consensus sequence (4, 10). We were unable to demonstrate rescue of the temperature-sensitive phenotype of these mutants with zinc salts added to agar (data not shown), but since the RNA polymerases are zinc metaloenzymes (20) and the similarity of this highly conserved region to the consensus sequence is so striking, it is likely that this region contains a zinc-binding site.

Regarding the zinc-binding consensus sequence, it is of interest that the *rpa190-5* mutation substituted a tyrosine for one of the conserved histidine residues (His-75), which has been suggested to be involved in coordinating a zinc atom (10, 23). Structurally, it is not known what the result of such a substitution would be, but it does not result in complete functional inactivation, as might be expected if an essential interaction is destroyed by the substitution (assuming that zinc binding is an essential requirement for enzyme activity). If this histidine residue is normally involved in coordinating zinc, the tyrosine substitution could possibly allow a compensatory interaction to occur at the permissive temperature that allows partial activity of the enzyme. Alternatively, it may be possible that His-75 may not be directly involved in coordinating zinc. We note that the 147-kDa RNA polymerase subunit from vaccinia virus, while otherwise highly similar to the eucaryotic RNA polymerase large subunits through region I, has a lysine at what would be the conserved His-78 position in the yeast polymerase I sequence (8). Also, most His-His pairs in protein sequences that are involved in the binding of zinc are separated by 3 or 4 amino acid residues (10). It should be noted that region I contains another invariant Cys-X_2-Cys pair, 24 amino acids downstream from the His-X_2-His pair, which could also be involved in a possible zinc-binding interaction. One of the temperature-sensitive mutations isolated in the yeast RNA polymerase II largest subunit affects a glycine residue adjacent to this Cys pair (12). While this hypothetical zinc-binding arrangement has 37 amino acid residues between the two Cys-X_2-Cys pairs, there is precedent for a long spacer between two closely spaced Cys pairs in the zinc domain of the regulatory chain of aspartate carbamoyltransferase, which contains a Cys-X_4-Cys-X_{23}-Cys-X_2-Cys sequence (4, 13). Thus, while the *rpa190-1* and *rpa190-5* mutations indicate that this region of the subunit carries out an important functional role, insight into the nature of this role will require further studies.

The *rpa190-3* mutation appears to be fully active at the permissive temperature and shows a tight temperature-sensitive phenotype at the nonpermissive temperature. The kinetics of RNA accumulation in this mutant after a shift to 37°C (Fig. 8) are similar to that seen when a strain in which the *RPA190* gene under the control of the glucose-repressible *GAL1* promoter is shifted from galactose to glucose medium (Wittekind, Ph.D. thesis). The in vitro activity of polymerase I in extracts made from *rpa190-3* cells harvested 8 h after the shift to 37°C was reduced by 80% relative to its activity in extracts made from *rpa190-3* cells grown at 23°C (Table 5). This phenotype can be explained by assuming that the *rpa190-3* allele encodes an A190 subunit with an assembly defect at the nonpermissive temperature. However, as mentioned above, the polymerase I activity from *rpa190-3* cells grown at 23°C appears to be heat labile in vitro. Apparently, such inactivation of polymerase I does not take place at 37°C in vivo, and the temperature-sensitive defect is manifested only gradually, as though the defect was in the synthesis of the enzyme and not in its activity.

The *rpa190-3* mutation falls within a small stretch of the amino acid sequence between the previously identified homology regions III and IV (8). This small region was not previously included in the conserved regions; but we found that it was very highly conserved among the largest subunits from RNA polymerases I, II, and III from yeasts and region II from mice (Fig. 6). This may be a sequence that is shared only among the largest subunits of eucaryotic nuclear RNA polymerases, since we failed to find homology to this region in the *E. coli* β′ and 147-kDa vaccinia virus RNA polymerase subunits, even though these subunits shared extensive homology with the mouse and yeast subunits in many other regions.

The isolation of a nonsense mutation in this screening was unexpected. An unsuppressed mutation of this type resulting in truncation of the A190 polypeptide at this point in the coding sequence would only leave approximately 40% of the N-terminal amino acid residues intact. In preliminary experiments, we purified the enzyme from this mutant grown at 23°C and found that the enzyme contained the apparently intact A190 subunit and had a thermal denaturation profile indistinguishable from that of the wild type. In addition, analysis of proteins synthesized after pulse-labeling of *rpa190-2* cells with [^{35}S]methionine revealed the presence of a radioactive peptide, which was immunoreactive to antibody specific for the A190 subunit and had the size expected for chain termination at the UGA codon. At 23°C, this peptide was detected together with the intact A190, whereas only the peptide, and no intact A190, was detected at 37°C. Thus, it appears that the strain has an UGA suppressor activity which is only partially functional at 23°C and that becomes ineffective at 37°C.

While in most eucaryotes the genes encoding the 5S RNA transcripts are unlinked to the rRNA genes, *S. cerevisiae* is unusual in that the gene for 5S RNA is included within the tandemly repeated ribosomal DNA repeat unit. The tight physical coupling of these genes raises the question of whether their expression is coupled. The hybridization results presented in Table 4 show that 5S RNA transcription by polymerase III in the nucleolus is relatively unaffected in cells in which the 35S RNA transcription rate by polymerase I is decreased by approximately 90% (see footnote *a* to Table 4). Therefore, the two transcriptional processes can be uncoupled.

All of the *rpa190* mutants isolated were unique, indicating that there are likely to be many more possible temperature-sensitive alleles. As discussed above, all three missense mutations that were sequenced changed amino acids that were highly conserved in all of the sequenced eucaryotic large-subunit RNA polymerase genes. Random mutations generated in the largest subunit gene of yeast RNA polymerase II have also affected amino acid residues that are invariant or else that lie within regions of high homology (12). Although the number of sequenced mutant alleles was statistically small, these results suggest that localized ran-

dom or site-directed mutagenesis of these highly conserved regions may be an efficient way of generating conditional mutations in these enzymes. It is likely that sequence information from mutations within a conserved region of one RNA polymerase may be used as a rational basis for introduction of mutations into the cognate subunit of another RNA polymerase via site-directed mutagenesis. This might allow the transfer of a particularly interesting mutation from one polymerase to another.

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