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Interaction between the Yeast Mitochondrial and Nuclear Genomes Influences the Abundance of Novel Transcripts Derived from the Spacer Region of the Nuclear Ribosomal DNA Repeat

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We have identified stable transcripts from the so-called nontranscribed spacer region (NTS) of the nuclear ribosomal DNA repeat in certain respiration-deficient strains of *Saccharomyces cerevisiae*. These RNAs, which are transcribed from the same strand as is the 37S rRNA precursor, are 500 to 800 nucleotides long and extend from the 5′ end of the 5S rRNA gene to three major termination sites about 1,780, 1,830, and 1,870 nucleotides from the 3′ end of the 26S rRNA gene. A survey of various wild-type and respiration-deficient strains showed that NTS transcript abundance depended on the mitochondrial genotype and a single codominant nuclear locus. In strains with that nuclear determinant, NTS transcripts were barely detected in [*rho*$^+$] cells, were slightly more abundant in various mit$^-$ derivatives, and were most abundant in petites. However, in one petite that was hypersuppressive and contained a putative origin of replication (ori5) within its 757-base-pair mitochondrial genome, NTS transcripts were no more abundant than in [*rho*$^+$] cells. The property of low NTS transcript abundance in the hypersuppressive petite was unstable, and spontaneous segregants that contained NTS transcripts as abundant as in the other petites examined could be obtained. Thus, respiration deficiency per se is not the major factor contributing to the accumulation of these unusual RNAs. Unlike RNA polymerase I transcripts, the abundant NTS RNAs were glucose repressible, fractionated as poly(A)$^+$ RNAs, and were sensitive to inhibition by 10 μg of α-amanitin per ml, a concentration that had no effect on rRNA synthesis. Abundant NTS RNAs are therefore most likely derived by polymerase II transcription.

Although the products of many nuclear genes and a few encoded by the mitochondrial genome are required for the synthesis of functional mitochondria, the molecular interactions between these organelles, which are required to achieve balanced mitochondrial synthesis, are still poorly understood. Expression of some nuclear genes encoding mitochondrial proteins is known to be regulated according to growth conditions and to various metabolic demands of the cell. In the yeast *Saccharomyces cerevisiae*, for example, growth of cells on high glucose concentrations represses the synthesis of nuclear-encoded components of the oxidative phosphorylation apparatus (55, 62). Yeast cells also have a regulatory network that adjusts the synthesis of mitochondrial heme proteins in response to fluctuations in the levels of heme (21). These regulatory pathways operate through interactions between *trans*-acting factors encoded at loci such as *HAP1*, *HAP2*, and *HAP3* and their target, *cis*-acting sites located upstream of the relevant genes (22, 44–46, 58).

Other nuclear genes have been identified which control mitochondrial gene expression in that their products exert specific control over mitochondrial RNA processing (3, 13, 14), mRNA stability (13–15), and translation (11, 47, 52). Although these genes and others like them could clearly serve to regulate mitochondrial biosynthesis as part of a nuclear-to-mitochondrial control circuit, there is little information on what form of regulation might occur in the other direction, from mitochondria to the nucleus.

Recently, we obtained evidence for a path of communication from mitochondria to the nucleus in yeast cells that might function in coordinate gene regulation (8, 40). The existence of such a path was suggested by the observation that the functional state of mitochondria, or the mitochondrial genotype, could influence the expression of a number of nuclear genes. Using cDNA subtraction procedures, we identified some transcripts of the nuclear genome whose abundance varied fivefold or more among derepressed isochromosomal cells in apparent response to respiration deficiency or to the quality or quantity of mitochondrial DNA (mtDNA). In that initial study, two classes of regulated transcripts were identified: those which were more abundant in respiration-deficient cells, mit$^-$ or petite, than in the respiratory competent [*rho*$^+$] parent (class I), and those which were more abundant specifically in petites (class II).

Among those differently regulated transcripts was a polydisperse group of RNAs that were especially interesting, since they were derived from the so-called nontranscribed spacer (NTS) region of the nuclear ribosomal DNA (rDNA) repeat. Those RNAs were barely detected in respiration-competent wild-type cells ([*rho*$^+$]) but were very abundant in [*rho*^{0}] petites, which lacked mtDNA. This result was surprising for at least two reasons. First, although it is now recognized that NTS sequences are transcribed in some organisms (9, 23, 25, 35, 36), those transcripts appear to be very unstable and have not been found to accumulate in cells to any appreciable extent. Second, the relationship between the relative abundance of NTS transcripts and the functional state of mitochondria or the mitochondrial genome was not immediately obvious.

To understand better the influence of mitochondria on nuclear gene expression and to assess the significance of the presence in some respiration-deficient yeast strains of abundant transcripts derived from the NTS, we have begun to characterize these unusual RNAs. Here, we provide evidence that the steady-state abundance of NTS transcripts

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TABLE 1. Yeast strains

Strain	Genotype		Mitochondrial function[a]
	Nuclear	Mitochondrial	
COP161			
Parent	a *ad lys*	[*rho*$^+$]	RC
E69	a *ad lys*	*mit*	RD, deletion in *oxi2*
C245	a *ad lys*	*mit*	RD, deletion in *oxi3*
PZ27	a *ad lys*	*mit*	RD, deletion in *cob*
HS40	a *ad lys*	[*rho*$^-$]	RD, hypersuppressive
F11	a *ad lys*	[*rho*$^-$]	RD
[*rho*^{0}]	a *ad lys*		RD
COP161 Ura$^-$			
Parent	a *ad lys ura*	[*rho*$^+$]	RC
[*rho*^{0}]	a *ad lys ura*		RD
22-2D			
Parent	α *ura trp leu* Chx^r Can^r	[*rho*$^+$]	RC
[*rho*^{0}]	α *ura trp leu* Chx^r Can^r		RD
MH41-7B			
Parent	a *ad his*	[*rho*$^+$]	RC
[*rho*^{0}]	a *ad his*		RD

[a] RC, Respiration competent; RD, respiration deficient.

depends on the mitochondrial genotype and a single nuclear locus. Our results are also consistent with the surprising conclusion that the abundant NTS RNAs are derived from RNA polymerase II rather than RNA polymerase I transcription.

MATERIALS AND METHODS

Strains and growth conditions. *S. cerevisiae* strains with different nuclear and mitochondrial genotypes used in this study are listed in Table 1. Cells were grown to mid-logarithmic phase in liquid YP medium (1% yeast extract, 1% Bacto-Peptone [Difco Laboratories, Detroit, Mich.]) containing 2% raffinose as the carbon source or as specified in the text. For nuclear run-on experiments, cells were grown on YN medium (0.67% yeast-nitrogen base plus amino acids and 3% raffinose). For genetic analysis, cells were mated on solid YP–2% glucose medium, prototrophic diploids were selected and sporulated, and tetrads were dissected by standard procedures. [*rho*^{0}] petites were isolated by three or more passages over YPD plates containing 20 μg of ethidium bromide per ml. A ura$^-$ derivative of COP161 (Table 1) was obtained by selection on 5-fluoro-orotic acid (6). Construction of particular combinations of mitochondrial and nuclear genotypes was carried out by cytoduction, using the *kar1* mutation as described by Nagley and Linnane (37).

RNA isolation. Total RNA was extracted from exponentially growing yeast cells by using two minipreparation methods. The method of Nasmuth (38) included the addition of 200 U of the RNase inhibitor RNasin (Promega Biotec) per ml to the extraction buffer. The hot phenol extraction method of Elion and Warner (16) was also used, with the spheroplasting step omitted. RNAs prepared by both methods were stored either as ethanol precipitates at −20°C or in 0.5% sodium dodecyl sulfate (SDS) at −70°C. Poly(A)$^+$ RNAs were prepared by chromatography on oligo(dT)-cellulose (1) or by selective binding and elution on Hybond-mAp paper as recommended by the manufacturer (Amersham Corp., Arlington Heights, Ill.).

Northern (RNA) blot analysis. Total or poly(A)$^+$ RNA was fractionated by electrophoresis on 1.25% agarose formaldehyde gels. After electrophoresis, RNA was transferred either to Hybond-N (Amersham) or to Zetaprobe membranes (Bio-Rad Laboratories, Richmond, Calif.). For riboprobe hybridizations, filters were prehybridized for 4 h, and RNA-RNA hybridization was carried out for 14 to 18 h in a hybridization buffer containing 50% (vol/vol) formamide, 0.75 M NaCl, 0.15 M Tris hydrochloride (pH 8.0), 0.01 M EDTA, 0.2 M $NaPO_4$ (pH 7.2), 0.5 mg of sonicated salmon sperm DNA per ml, 0.1% (wt/vol) SDS, and 0.1% (wt/vol) each bovine serum albumin, Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), and polyvinylpyrrolidone at 55°C. Northern blots were washed once in 0.75 M NaCl–0.15 M Tris hydrochloride (pH 8.0)–0.01 M EDTA–0.02 M $NaPO_4$ (pH 7.2)–0.1% (wt/vol) SDS–0.1% (wt/vol) sodium pyrophosphate at 55°C for 30 min, once in 0.15 M NaCl–0.03 M Tris hydrochloride (pH 8.0)–0.002 M EDTA–0.02 M $NaPO_4$ (pH 7.2)–0.1% SDS–0.1% (wt/vol) sodium pyrophosphate at 55°C for 30 min, and once in 0.05 M NaCl–0.005 M Tris hydrochloride (pH 8.0)–0.6 mM EDTA–0.02 M $NaPO_4$ (pH 7.2)–0.1% (wt/vol) sodium pyrophosphate–0.1% SDS at 55°C for 30 min. The dried blots were then exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) for autoradiography. Hybridizations using DNA probes were carried out for 14 to 18 h in hybridization buffer containing 50% (vol/vol) formamide, 0.74 M NaCl, 0.5 M NaH_2PO_4, 0.005 M EDTA (pH 7.4), 0.1% (wt/vol) each bovine serum albumin, Ficoll, and polyvinylpyrrolidone, 1% SDS, and 100 μg of sonicated calf thymus DNA per ml. After hybridization, the blots were washed once for 30 min at 65°C in 0.3 M NaCl–0.03 M sodium citrate (2× SSC)–0.5% SDS. RNA loads were normalized to the signal level of actin mRNA by using the appropriate actin riboprobe or DNA probe (see below). These probes were either mixed with other probes, used in separate experiments to determine actin mRNA levels, or used after removal of the original probe by washing the blot three times in boiling 0.1× SSC–0.15% SDS, followed by a rinse at room temperature in the same buffer.

Preparation of probes. The original 200-base-pair (bp) [*rho*19] cDNA (40) was subcloned from pBR322 by digestion with *Pst*I and ligated into the single *Pst*I site in the polylinker of pGEM4 (Promega). The orientation of the fragment from one of the clones was determined by sequencing the pGEM vector with use of a primer to the T7 promoter. RNA probes to [*rho*19] were generated by using T7 RNA Polymerase and plasmid linearized with *Eco*RI. An RNA probe to actin was prepared by subcloning an internal *Cla*I fragment of the actin gene derived from the pYact1 plasmid containing the entire actin gene (39) into pUC18, followed by removal of the 600-bp actin fragment by digestion with *Eco*RI and *Hin*dIII and ligation of the fragment into pGEM4 *Eco*RI and *Hin*dIII polylinker sites. The actin probe was generated by using Sp6 RNA polymerase and plasmid linearized with *Hin*dIII. All riboprobes were labeled with [α-^{32}P]UTP. Labeled DNA probes were prepared by nick translation of these same [*rho*19] and actin fragments, using [α-^{32}P]dCTP. To prepare single-stranded M13 clones, a 1.1-kbp *Bgl*II-*Eco*RI fragment internal to the 26S rRNA gene was subcloned into M13mp18. The single-stranded [*rho*19] probe was cloned into the *Hin*dIII and *Eco*RI sites of M13mp18. A Ty*1*-specific probe was made by subcloning a 0.75-kbp *Pst*I fragment, which overlaps Ty*A* and Ty*B* (10), into the single *Pst*I site in M13mp18. A TCM-1 probe was made by subcloning a 1.6-kbp *Pvu*II fragment from plasmid pTCM 3.2 (19) into

pGEM-3Zf(+) (Promega). In all cases, orientation of the M13 subclones was determined by restriction analysis.

S1 nuclease mapping. A 1,018-nucleotide (nt) *Hin*dIII-*Ava*II fragment end labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase and a 1,062-nt *Taq*I fragment labeled by filling in sticky ends with [α-^{32}P]dCTP and Klenow fragment of DNA polymerase were used for 5′ and 3′, respectively, mapping of NTS transcripts. The labeled fragments were denatured by heating at 90°C for 2 min in 0.05 M NaOH–0.01 M EDTA–10% sucrose. After quick cooling on ice, the denatured single strands were separated by electrophoresis on a 6% acrylamide-urea gel at 40°C. The full-length separated fragments were located by autoradiography, cut out, and electroeluted from the gel as described previously (61). The appropriate single-stranded fragment (2,000 to 10,000 cpm) and about 5 μg of poly(A)$^+$ RNAs from [*rho*$^+$] and [*rho*^{0}] strains were precipitated together with ethanol, and the precipitates were dried and dissolved in 25 μl of buffer containing 0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.001 M EDTA, 0.4 M NaCl, and 45% formamide. After heating at 65°C for 5 min, hybridizations were carried out for 16 h at 42°C. After 16 h of incubation, 200 μl of S1 nuclear buffer (0.28 M NaCl, 0.03 M sodium acetate [pH 4.6], 0.0045 M zinc acetate, 40 μg of heat-denatured herring sperm DNA) was added. Digestions were initiated by the addition of 200 U of S1 nuclease and were continued at 37°C for 30 min. Reactions were terminated by the addition of 30 μl of 2.5 M ammonium acetate, 0.05 M EDTA, and 20 μg of carrier tRNA, and the nucleic acids were precipitated with 3 volumes of ethanol. The resultant pellet was washed with 80% ethanol, dried, and dissolved in 20 μl of deionized formamide plus 0.03% bromophenol blue, 0.03% xylene cyanol, 1 mM EDTA, and 5% sucrose. Before electrophoresis, samples were first heated to 90°C for 5 min and then placed on ice. The samples were separated by electrophoresis on an 8% polyacrylamide sequencing gel.

Primer extension analysis. A 22-base oligonucleotide (5′-GCGAAACTCAGGTGCTGCAATC-3′) complementary to [*rho*19] RNA was labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase. About 5 μg of poly(A)$^+$ RNA from COP161 [*rho*$^+$] and [*rho*^{0}] strains was annealed with 1×10^6 to 2×10^6 cpm of the 22-mer in reverse transcriptase buffer (50 mM Tris [pH 8.0], 150 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 7 mM $MgCl_2$) for 2 min at 65°C, followed by quick cooling on ice for 5 min. After annealing, 40 U of reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) was added together with 0.5 mM deoxynucleoside triphosphates and incubated at 42°C for 90 min. RNA was removed by addition of NaOH to 0.1 M and heating for 3 min at 90°C. After neutralization, the labeled cDNAs were analyzed by electrophoresis on 8% polyacrylamide-urea gels.

Nuclear run-on experiments using permeabilized yeast cells. Intact yeast cells were permeabilized with 0.5% (wt/vol) *N*-laurosarcosine, and nuclear run-on transcription was carried out as described by Elion and Warner (17). The ^{32}P-labeled RNA was hybridized to single-stranded M13 probes immobilized on nitrocellulose filters prehybridized in 50% formamide–0.36 M NaCl–0.02 M $NaPO_4$ (pH 7.7)–0.002 M EDTA–0.1% SDS–0.1 mg of sonicated salmon sperm DNA per ml. Hybridization was for 66 to 72 h at 42°C. Filters were washed as described by Elion and Warner (17), with two additional washes at room temperature for 15 min in 2× SSC, followed by incubation for 30 min at 37°C in 2× SSC containing 5 μg of RNase A per ml. Filters were blotted dry and exposed to Kodak X-Omat film for autoradiography.

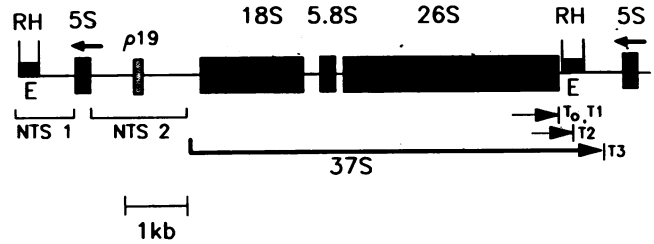

FIG. 1. Organization of the *S. cerevisiae* nuclear rDNA repeat unit. Locations of the 18S, 5.8S, 26S, and 5S rRNA genes are indicated. The stippled box represents the region of the rDNA repeat with homology to the [*rho*19] cDNA clone. The 5S rRNA gene and the 37S rRNA precursors are transcribed in opposite directions (arrows). Restriction endonuclease sites: R, *Eco*RI; H, *Hin*dIII. Positions of the major processing-termination sites are indicated as T_0 to T_3 (27). The enhancer region (E) is included within these restriction sites (16).

RESULTS

Transcripts from the nuclear rDNA spacer are regulated and transcribed from the same strand as is the 37S rRNA precursor. Among the cDNAs obtained in the subtractive hybridizations between various respiration-deficient strains, one, designated [*rho*19], had perfect homology to a region of the NTS located between the 5S rRNA gene and the start of transcription of the 37S rRNA precursor (Fig. 1). Since that region of the rDNA repeat in yeast cells appears to be well beyond the documented limits of the major initiation, termination, or processing sites for both the 5S rRNA and the 37S rRNA precursor transcripts (27, 28, 57), it was quite surprising to find that some cells contained abundant amounts of these NTS RNAs.

The 5S rRNA and 37S rRNA precursor in *S. cerevisiae* are transcribed from opposite strands of the rDNA repeat by RNA polymerases III and I, respectively (57). To determine the direction of transcription of the major regulated NTS RNAs, ^{32}P-labeled strand-specific riboprobes were generated from the original ~200-bp [*rho*19] cDNA, which was subcloned for that purpose into the *Pst*I site of a pGEM vector containing bacteriophage Sp6 and T7 RNA polymerase promoters. These riboprobes were used in Northern blot hybridizations to total RNA isolated from the four isochromosomal strains described in our previous study (16; Table 1): a respiration-competent [*rho*$^+$] strain (COP161) and three respiration-deficient derivatives, a mit$^-$ (E69) harboring a deletion in the coxIII gene, a hypersuppressive [*rho*$^-$] petite (HS40), and a [*rho*^{0}] petite (Fig. 2). The HS40 [*rho*$^-$] strain was selected among ethidium bromide-induced petite isolates of COP161 on the basis of its suppressivity (>95%) when crossed to [*rho*$^+$] testers. As is characteristic of such petites (5, 12), HS40 contains a putative origin of mtDNA replication, in this case ori5 (12), within its 757-bp mitochondrial genome (data not shown). Using a [*rho*19] riboprobe antisense to the 37S rRNA precursor RNA, a strong hybridization signal to a set of polydisperse RNAs centering on two prominent bands of approximately 800 and 500 nt long was observed (Fig. 2A). Consistent with our previous results, these NTS transcripts were most abundant in the [*rho*^{0}] strain and were barely detected in the [*rho*$^+$] strain (Fig. 2). (In all experiments of this type, the RNA loads were normalized to the level of the 1.4-kbp actin mRNA, since the abundance of that RNA species is not significantly different in [*rho*$^+$] and respiration-deficient strains [40].) Using the complementary [*rho*19] riboprobe, which is of the same

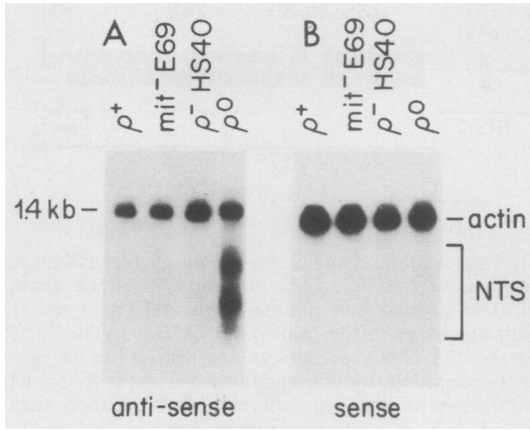

FIG. 2. Regulation and transcription of NTS transcripts from the same strand that regulates the 37S rRNA precursor. [α-^{32}P]UTP-labeled riboprobes of the same sense (B) and antisense (A) to the 37S rRNA precursor were synthesized from the linearized pGEM [*rho*19] vector as described in Materials and Methods. The probes were hybridized to Northern blots of total RNA isolated from COP161 [*rho*$^+$], E69, HS40 and [*rho*^{0}] strains. The amount of RNA in each sample was normalized to the signal obtained with a riboprobe to actin mRNA.

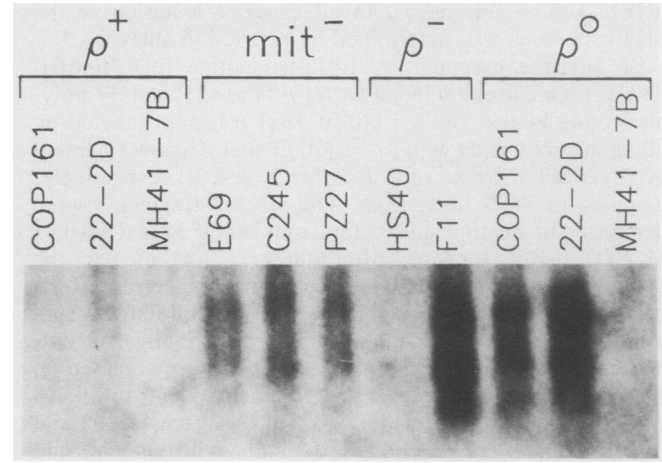

FIG. 3. Northern blot analysis of NTS transcripts in strains with different mitochondrial and nuclear genotypes. Total RNA from the strains shown (see also Table 1) was fractionated on 1.25% agarose-formaldehyde gels, transferred to Hybond-N membranes and hybridized with a [*rho*19] cDNA probe. The amount of RNA in each lane was normalized to the signal given by actin mRNA.

sense as the 37S precursor rRNA, only weak hybridization signals were observed (Fig. 2B). Since the in vitro transcription reactions with these vectors are not absolutely strand specific (34), these latter faint signals were most likely due to a small amount of antisense probe generated in the in vitro transcription reaction. From these experiments, we conclude that the abundant NTS RNAs are transcribed from the same strand as is the 37S rRNA precursor.

The abundance of NTS transcripts depends on both the mitochondrial and the nuclear context. The striking difference in the amount of NTS transcripts between the [*rho*^{0}] petite and the other respiration-deficient strains raised the question of whether these RNAs are peculiar to [*rho*^{0}] petites, that is, are expressed only in the absence of mtDNA. To answer this question, we surveyed other respiration-deficient strains derived from COP161 [*rho*$^+$], each with a different and well-defined lesion in the mitochondrial genome (Table 1). These included the petite, F11, which harbors a 12-kbp mitochondrial genome containing the entire 21S rRNA gene and some downstream tRNA genes (31), and two mit$^-$ strains, one (PZ27) with a deletion in the cytochrome *b* gene (42) and the other (C245) with a deletion in the coxI gene (32). Like the mit$^-$ E69 strain (Fig. 1), the mitochondrial genomes of PZ27 and C245 were stable in that the frequency of spontaneous petites was about the same as that of the [*rho*$^+$] parent (<1%). In addition to these COP161 derivatives, we analyzed NTS abundance in [*rho*$^+$] and [*rho*^{0}] derivatives of two other strains with different nuclear backgrounds, MH41-7B and 22-2D (Table 1).

Northern analysis of total RNA from these strains showed a striking dependence of NTS transcript abundance on both the mitochondrial genotype and the nuclear background (Fig. 3). Among the six respiration-deficient derivatives of COP161 [*rho*$^+$], NTS transcripts were most abundant in F11, whereas they were barely detected in the hypersuppressive petite, HS40. The three mit$^-$ strains, although having different lesions in the mitochondrial genomes, contained comparable amounts of NTS RNAs, which were somewhat more abundant than in the [*rho*$^+$] strains but significantly less abundant than in the F11 or [*rho*^{0}] petite strains.

A number of important conclusions can be drawn from these results. First, it is clear that the accumulation of NTS transcripts is not simply a consequence of respiration deficiency, as was previously suggested (8, 40). Second, the absence of mtDNA per se cannot account for elevated NTS expression, since we have consistently observed that NTS RNAs are most abundant in the petite, F11. Third, not only is NTS abundance affected by the mitochondrial genotype, but it is also dependent on the nuclear context, as is evident from a comparison of [*rho*$^+$] and [*rho*^{0}] derivatives of strains COP161, MH41-7B, and 22-2D (Table 1). Whereas NTS RNAs were comparably abundant in the [*rho*^{0}] derivatives of COP161 and 22-2D, they were not detectable in the [*rho*^{0}] derivative of MH41-7B (Fig. 3).

The variation in NTS RNA abundance among isochromosomal petites is further demonstrated by the experiment shown in Fig. 4. A spontaneous segregant of the hypersuppressive petite, HS40, which no longer showed the hypersuppressive phenotype when crossed to a [*rho*$^+$] tester strain (data not shown), was selected. A comparison of the amount of NTS transcripts in this segregant with the amount in the original HS40 isolate shows that NTS RNAs were now just as abundant as in the original [*rho*^{0}] strain. Over the course of many similar experiments, we have noted that the property of low NTS transcript abundance in the HS40 petite is an unstable characteristic of that strain: over time, cultures of HS40 tend to show levels of NTS RNAs approaching that of the [*rho*^{0}] petite. In preliminary experiments to examine this phenomenon, we have not been able to correlate the instability in any direct way with the loss of mtDNA, loss of hypersuppressivity, or any major rearrangements of the HS40 genome; other, more subtle changes in the HS40 genome have not been ruled out and remain a distinct possibility to account for this drift.

NTS abundance in petites depends on a single nuclear locus. The results presented above indicated that NTS expression is determined not only by the mitochondrial genotype but also by the nuclear context. To determine the genetic complexity of this effect, five tetrads obtained from a cross between strains 22-2D and MH41-7B were analyzed by Northern analysis for the abundance of NTS RNAs after

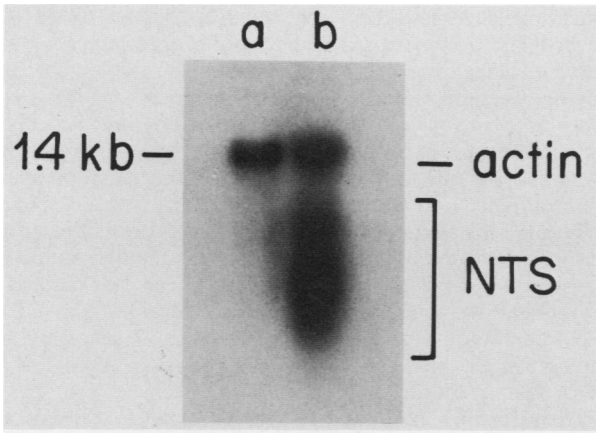

FIG. 4. Appearance of abundant NTS transcripts in a spontaneous, neutral (nonhypersuppressive) segregant of the hypersuppressive petite, HS40. A spontaneous segregant of HS40 that no longer showed a hypersuppressive phenotype when crossed to [*rho*$^+$] testers was randomly selected. Total RNA from this segregant (lane b) and from the original HS40 petite (lane a) was analyzed by Northern blot analysis for the abundance of NTS transcripts as described in Materials and Methods.

conversion of the meiotic segregants to [*rho*^{0}] petites by growth on ethidium bromide. The results obtained for all five tetrads, two examples of which are presented in Fig. 5, showed that the pattern of abundant NTS transcripts segregated 2:2 among the [*rho*^{0}] spores, which indicated that a single nuclear locus is involved in determining NTS expression in a [*rho*^{0}]petite. Inspection of the levels of the NTS transcripts in a [*rho*^{0}] diploid derived from this cross suggested further that the nuclear locus affecting NTS transcript abundance is codominant.

5′ and 3′ mapping of NTS RNAs. To delimit the boundaries of the abundant NTS transcripts, we used a combination of primer extension and S1 nuclease protection analysis on poly(A)$^+$ RNA isolated from COP161 [*rho*^{0}] cells and from [*rho*$^+$] strain used as a control. As described below, we found that these NTS transcripts fractionated on oligo(dT)-cellulose columns, as is typical poly(A)$^+$ RNAs. Therefore, to increase the sensitivity of the S1 nuclease and primer extension analyses, the experiments described here were carried out with poly(A)$^+$ RNA. To map 5′ ends of primer extension, a 5′-^{32}P-labeled 22-nt oligomer complementary to NTS RNA was used as a primer (Fig. 6D). Analysis of the labeled, primer-extended cDNAs (Fig. 6A) showed first that RNA from the [*rho*^{0}] strain gave rise to a number of fragments, whereas only faint bands were observed after using an equivalent amount of RNA from the [*rho*$^+$] strain. This result, and those described below, are fully consistent with the abundance measurements of NTS transcripts determined by Northern analysis. Second, primer extension of RNA templates form the [*rho*^{0}] strain yielded a major 425-nt fragment corresponding to a 5′ RNA terminus located close to the 5′ border of the 5S rRNA gene, about 1,230 bp from the 3′ end of the 26S rRNA (Fig. 1), and a series of less prominent fragments, mostly shorter in length.

Since primer-extended fragments can arise form pausing at RNA secondary structures during reverse transcription as well as from run-off from RNA ends, we carried out S1 nuclease protection experiments on the same RNAs by using a 1,018-nt *Hin*dIII-*Ava*II probe (Fig. 6D). In this experiment (Fig. 6B), a major 41-nt S1-protected fragment, corresponding to a 5′ RNA end at about position 1260 from the 3′ end of the 26S rRNA, was observed only with the RNA from the [*rho*^{0}] strain, in reasonable agreement with the major 5′ end estimated by primer extension. In both the primer extension and the S1 nuclease protection experiments, long exposures of the gels (not shown) revealed larger fragments that would correspond to 5′ RNA ends located between the 5S rRNA gene and the enhancer region (Fig. 1).

To map the 3′ ends of the NTS RNAs, S1 nuclease protection analysis was carried out with 5′-end-labeled coding and noncoding strands of a 1,062-bp *Taq*I fragment (Fig. 6D) as probes and the same poly(A)$^+$ RNA from COP161 [*rho*$^+$] and [*rho*^{0}] as was used above. This experiment (Fig. 6C) showed that the NTS coding-strand probe and RNAs from the [*rho*^{0}] strain gave rise to three S1-protected fragments corresponding to termini at about 1,781, 1,832, and 1,870 nt from the 3′ end of the 26S rRNA gene.

These transcript mapping data, summarized diagrammatically in Fig. 7, predict major NTS RNAs ranging in length

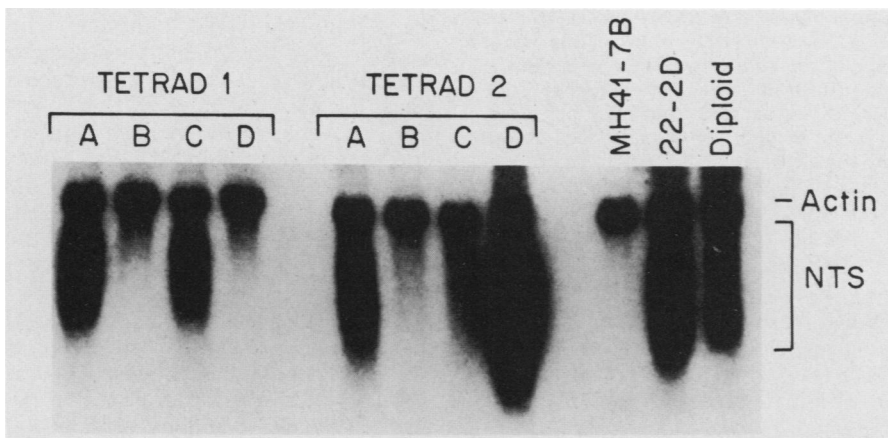

FIG. 5. Influence of a single codominant nuclear locus on accumulation of NTS transcripts. Strains 22-2D and MH 41-7B were crossed, diploids were sporulated, and tetrads were dissected by standard procedures. Total RNA isolated from [*rho*^{0}] derivatives of each of the spores, from the parental haploids, and from the diploids was fractionated on 1.25% agarose-formaldehyde gels, transferred to Hybond-N membranes, and hybridized with [*rho*19] and actin riboprobes as described in Materials and Methods. Shown are results for two tetrads from a total of five analyzed, along with results for the parental haploid and diploid strains.

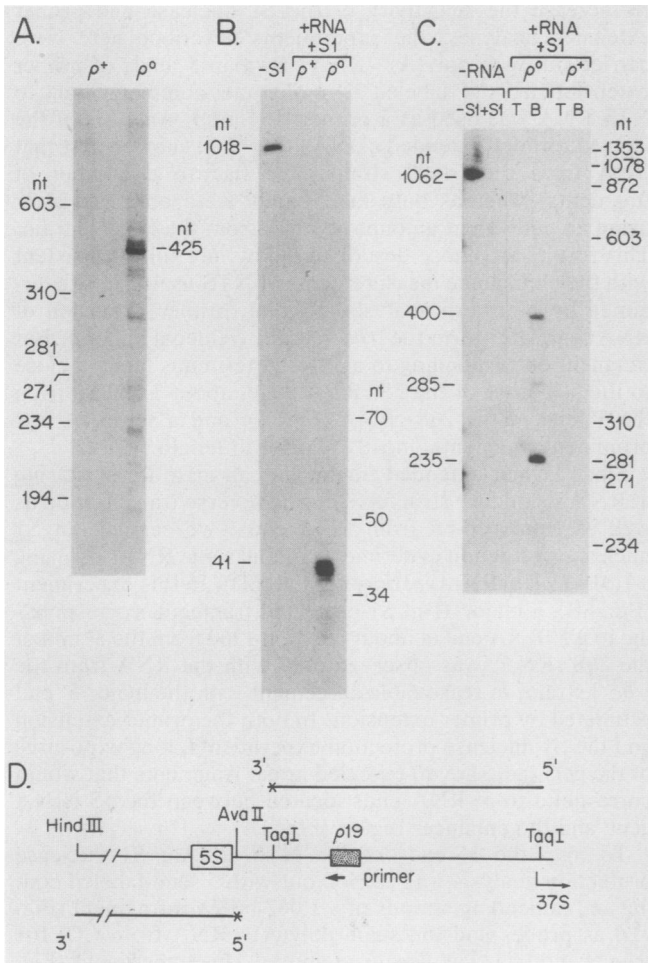

FIG. 6. Primer extension and S1 nuclease analysis of NTS transcripts. (A) Primer extension. A 5'-end-labeled 22-mer (see Materials and Methods) complementary to [*rho*19] transcripts was hybridized to poly(A)$^+$ RNA from COP161 [*rho*$^+$] and [*rho*^{0}] and extended with reverse transcriptase as described in Materials and Methods. Reaction products were analyzed on 8% acrylamide-urea gels. The sizes of the major primer-extended products were estimated from the mobilities of *Hin*fI fragments of ΦX174. (B) Mapping of the 5' ends of NTS RNAs by S1 nuclease. A 5'-end-labeled 1,018-nt *Hin*dIII-*Ava*II fragment from the rDNA repeat (panel D) was hybridized to 5 μg of poly(A)$^+$ RNA from COP161 [*rho*$^+$] and [*rho*^{0}]. (C) Mapping of 3' ends. A 1,062-bp *Taq*I fragment (panel D) was 3' end labeled by being filled in with [α-^{32}P]dCTP and Klenow fragment. The NTS coding strand was isolated from a 5% polyacrylamide gel and hybridized to the poly(A)$^+$ RNAs described above. Controls without RNA or S1 nuclease are indicated. T, Top (RNA-like) strand; B, bottom (template) strand. After reaction with S1 nuclease, the products were analyzed on an 8% polyacrylamide-urea gel as described in Materials and Methods. *Hin*fI fragments of ΦX174 served as size markers.

from about 500 to 650 nt, in reasonable agreement with the size estimates obtained from Northern analysis. At their extremes, none of the NTS transcripts fell within any known termination, processing, or initiation sites for the 37S rRNA precursor (4, 27, 28).

NTS transcripts are glucose repressible. To minimize the possibility that detection of differently regulated nuclear DNA sequences would be obscured by glucose repression, all of the experiments carried out in these and in our previous study (8, 40) used cells that were grown on the fermentable but nonrepressing sugar, raffinose. Since the rate of RNA polymerase I transcription, and hence rRNA synthesis, is maximal in *S. cerevisiae* cells grown on glucose (29, 56), we anticipated that the abundance of NTS transcripts in a [*rho*^{0}] petite grown on glucose would be either unaffected or elevated even further in comparison with the abundance in the same cells grown on the nonrepressing sugar, raffinose. To examine this possibility, we compared NTS transcript abundance in COP161 [*rho*^{0}] cells grown to mid-logarithmic phase in medium containing 10% glucose, 2% galactose, a mildly repressing sugar, or 2% raffinose. Surprisingly, we found that the NTS transcripts behaved as typical catabolite-repressible RNAs, since they were least abundant in the glucose-grown cells and most abundant in cells grown on raffinose (Fig. 8).

NTS transcripts fractionate as typical poly(A)$^+$ RNAs. In our initial cDNA subtraction screen to detect differently regulated transcripts, cDNAs were generated by oligo(dT) priming of poly(A)$^+$ RNA fractions obtained from the various respiration deficient strains. Although the 200-bp NTS cDNA, [*rho*19], was obtained from this screen, no particular significance was given at that time to the fact that it was generated from poly(A)$^+$ RNA. The results presented above, showing that NTS RNAs in a [*rho*^{0}] petite were highly glucose repressible, an observation inconsistent with the known behavior of RNA polymerase I transcription in *S. cerevisiae* (29, 56), prompted us to determine whether the abundant NTS RNAs might be polyadenylated. Total RNA was isolated from COP161 [*rho*^{0}] grown on 2% raffinose and fractionated over oligo(dT)-cellulose to obtain poly(A)$^+$ and poly(A)$^-$ RNA fractions. As a control, the distribution of actin mRNA, a typical polyadenylated RNA, was determined on the same RNA samples. A Northern blot of the various RNA fractions from the oligo(dT)-cellulose column, using actin and NTS [*rho*19]) probes, showed, remarkably, that the NTS RNAs fractionated exactly as did the polyadenylated actin mRNA (Fig. 9), which indicated that these abundant NTS transcripts were polyadenylated.

In higher eucaryotes, processing and polyadenylation of most RNA polymerase II transcripts occurs in response to the sequence 5'-AAUAAA-3', usually found 10 to 30 nt upstream of the major polyadenylation site (18, 60). Although that sequence or versions of it are apparently not used for the same purpose in *S. cerevisiae*, a consensus tripartite sequence (Fig. 10) has been found in the vicinity of the polyadenylation site(s) of many yeast RNA polymerase II genes (48, 53, 59). It has been suggested that this tripartite element is a yeast version of a processing-polyadenylation signal for RNA polymerase II transcripts. Inspection of the DNA sequence in the vicinity of the putative polyadenylation sites of the major NTS transcripts reveals a sequence very similar to the consensus tripartite sequence (Fig. 10). Moreover, the position of that sequence relative to the 3' NTS polyadenylation sites is similar to that observed for a number of other typical yeast RNA polymerase II transcripts.

RNA polymerase II transcription of the NTS. The results presented above strongly suggested that the stable NTS RNAs originated from RNA polymerase II rather than by RNA polymerase I transcription. To examine this possibility further, we used detergent-permeabilized yeast cells to determine the effects of the specific RNA polymerase II inhibitor, α-amanitin, on transcripts derived from the NTS region. As shown by Elion and Warner (17), such cells provide a convenient way to examine nuclear run-on transcription in vivo. RNA extracted from permeabilized [*rho*$^+$]

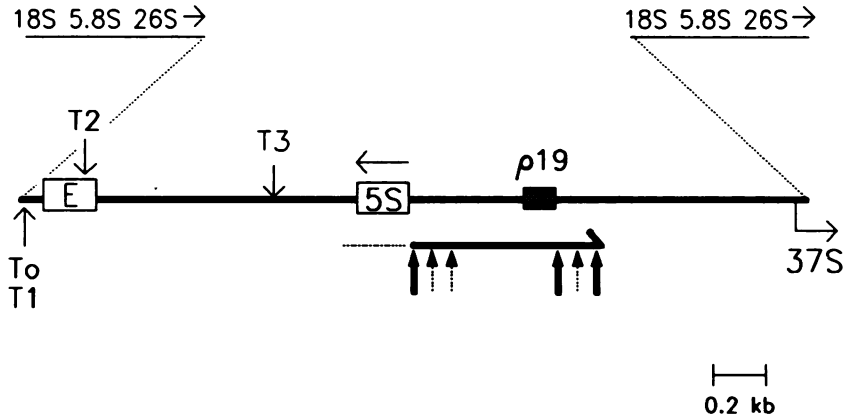

FIG. 7. Mapping of stable NTS transcripts. A portion of the rDNA repeat from the enhancer (E) to the start of transcription of the 37S rRNA precursor is shown. Location of the abundant NTS RNAs is indicated by the heavy horizontal arrow; less abundant NTS RNAs (dotted line) extend past the 5S rRNA gene. Heavy vertical arrows indicate major 5′ and 3′ termini. The hatched box shows the region contained within the original [*rho*19] cDNA.

and [*rho*^{0}] COP161 cells pulse-labeled for 10 min in the absence and presence of 10 and 250 μg of α-amanitin per ml was hybridized to immobilized single-stranded M13 probes specific for NTS transcripts (derived from the [*rho*19] cDNA), the 26S rRNA, and transcripts from two highly expressed RNA polymerase II genes, TCM-1, which encodes the cytoplasmic ribosomal protein L3 (19), and the yeast transposable element Ty*1* (Fig. 11). As expected, 26S rRNA synthesis was relatively insensitive to inhibition by 250 μg of α-amanitin per ml, whereas synthesis of the TCM-1 and Ty*1* transcripts was inhibited to background levels by 10 μg of the inhibitor per ml. Significantly, that concentration of inhibitor also reduced incorporation into NTS transcripts to background levels. Taken together, these data strongly suggest that the NTS RNAs are derived, at least in part, by polymerase II transcription.

DISCUSSION

Our previous study (40), establishing that some nuclear DNA sequences are expressed differently among different respiration-deficient yeast strains, was limited to three derivatives of COP161 [*rho*$^+$]: a mit$^-$ strain, a hypersuppressive petite, and a petite lacking mtDNA. One of the goals of this work was to establish the generality of this novel interaction between organelles by extending those observations to include other respiration-deficient cells with different lesions in the mitochondrial genomes and to strains with different nuclear backgrounds.

As a test system for these experiments, we examined transcripts from the NTS region of the nuclear rDNA repeat. Although other more conventional or even possibly more relevant regulated transcripts were identified in that initial study, such as those from the cytochrome oxidase subunit

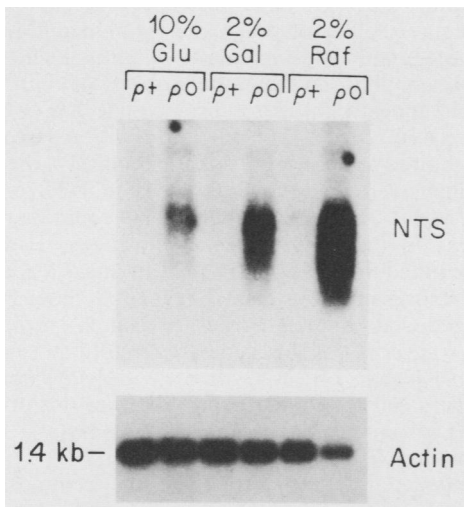

FIG. 8. Evidence that NTS transcripts are glucose repressible. Total RNA from COP161 [*rho*$^+$] and [*rho*^{0}] cells grown to mid-logarithmic phase on 10% glucose (Glu), 2% galactose (Gal), or 2% raffinose (Raf) was analyzed for NTS transcript abundance by Northern blot analysis as described in Materials and Methods. RNA loads were normalized to the amount of actin mRNA shown in the bottom panel.

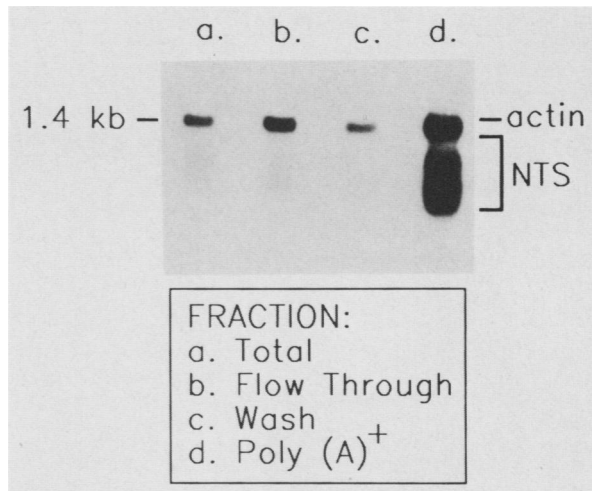

FIG. 9. Fractionation of NTS transcripts as poly(A)$^+$ RNAs. Total RNA from COP161 [*rho*^{0}] was fractionated on an oligo(dT)-cellulose column essentially as described by Aviv and Leder (1). Equal amounts (5 μg) of RNA from the total (a), flowthrough (b), wash (c), and (d) poly(A)$^+$ fractions were fractionated on a 1.25% agarose-formaldehyde gel. RNAs were transferred to Hybond-N membranes and probed with ^{32}P-labeled [*rho*19] and actin riboprobes.

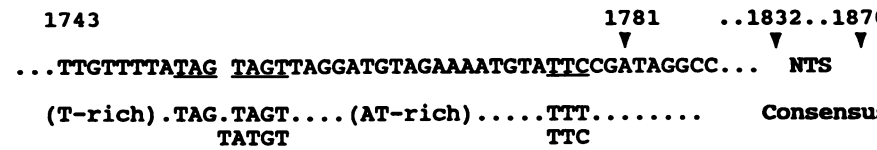

FIG. 10. A consensus of a tripartite polymerase II polyadenylation-processing sequence (59) near the 3′ ends of the major NTS RNAs. Arrows indicate positions relative to the 3′ ends of the 26S rRNAs of the 3′ ends of the major NTS transcripts.

VI gene (40), we have focused on the NTS transcripts for several reasons. First, the differences in abundance of these RNAs among the various strains are as great as, if not greater than, differences in any of the differently expressed sequences we have identified thus far. This fact could provide a convenient experimental system to analyze the molecular mechanisms by which the state of mitochondria or the mitochondrial genome may influence nuclear gene expression. Second, and in connection with this first point, since NTS transcripts not only are class II (petite-specific) RNAs but are expressed differently among isochromosomal petite isolates, the interesting possibility is raised that specific mtDNA sequences, or some particular mitochondrial genome organization, may be involved in signaling to the nucleus. Finally, the detection of abundant and differently regulated transcripts from the NTS region of the nuclear rDNA repeat was a completely unexpected finding; accumulation of such stable transcripts in cells is, to our knowledge, unprecedented and thus warrants further study from the standpoint of both rRNA gene expression and possible unexplored relationships to nuclear-mitochondrial interactions.

NTS expression depends on the mitochondrial genome and a single nuclear locus. Our survey of various *S. cerevisiae* strains with different mitochondrial and nuclear genotypes has allowed us to define more clearly the phenomenon that the state of the mitochondrial genome can influence expression of nuclear genes. For NTS transcripts, respiration deficiency appears necessary but not sufficient to account for elevated levels of the NTS RNAs. The three mit$^-$ strains derived from COP161 [*rho*$^+$] with defects in the *oxi2*, *oxi3* and *cob* genes differ in their mtDNA lesions, but the level of NTS transcripts in those cells is about the same and only slightly higher than the level in the [*rho*$^+$] parent. A comparison of the three petite strains derived from COP161, however, shows clear-cut differences in the levels of NTS transcripts: those RNAs are consistently more abundant in the petite F11, which contains a 12-kbp mitochondrial genome that includes the complete 21S rRNA gene and a number of downstream tRNA genes (31), and are very low in isolates of a hypersuppressive petite, HS40, containing a 757-bp mitochondrial genome repeat unit. Since the COP161 [*rho*^{0}] petite, which lacks any mtDNA, also contains abundant NTS transcripts, though not to the same extent as does F11, the conclusion is clear that among respiration-deficient *S. cerevisiae* cells, the nuclear genome must in some way sense these qualitative and quantitative differences in the mitochondrial genome. Interestingly, the property of low NTS transcript abundance in the HS40 petite is relatively unstable, since spontaneous segregants that contain NTS transcripts as abundant as in the [*rho*^{0}] petite can be isolated. Cultures of the original HS40 isolate tend to accumulate such segregants even in rich medium, which suggests that they may have some selective advantage. We have not yet established any molecular correlates which might account for this instability except that it is not necessarily associated with loss of hypersuppressivity or any apparent major rearrangements of the HS40 genome (R. Docherty, unpublished observations).

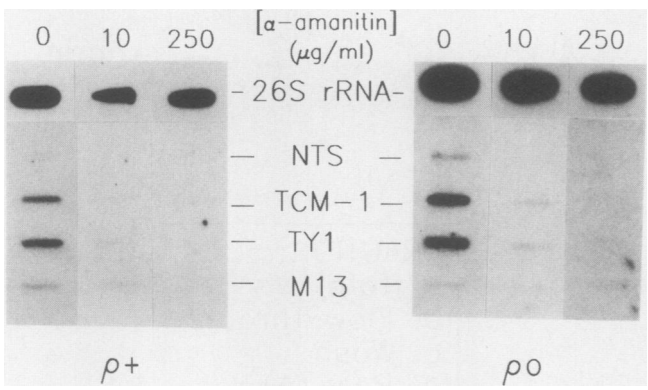

FIG. 11. α-Amanitin sensitivity of NTS transcripts. Sensitivity of the NTS transcripts was determined by using the nuclear run-on method of Elion and Warner (17). The equivalents of (i) 1 μg of single-stranded M13 probes to the 26S rRNA, TCM-1, and Ty*1* transcripts and of (ii) 0.5 μg of [*rho*19] and 25 μg of M13mp18 were immobilized on nitrocellulose filters and probed with RNA extracted from permeabilized [*rho*$^+$] and [*rho*^{0}] derivatives of COP161 Ura$^-$ (Table 1) labeled for 10 min with [α-^{32}P]UTP in the absence and presence of 10 or 250 μg of α-amanitin per ml.

These results also show that NTS expression is dependent on a single codominant nuclear locus. In contrast to strains COP161 and 22-2D, we have not observed NTS expression in any petite isolate of MH41-7B examined thus far. In preliminary genetic experiments to map this locus (H. Conrad-Webb, unpublished data), the property of abundant NTS transcripts appears linked to chromosome XII, which contains the rDNA repeat (43). Although additional experiments will be required to determine linkage precisely, the possibility remains open that the differences in NTS expression between MH41-7B and the other strains could be related to differences in the rDNA repeat sequences. However, it should be noted that MH41-7B [*rho*^{0}] strains also fail to show elevated expression of a class I regulated gene, MRP13, a nuclear-encoded mitochondrial ribosomal protein (41), whereas transcripts of that gene are fivefold more abundant in a [*rho*^{0}] isolate of 22-2D than in its [*rho*$^+$] parent (T. Mason, personal communication). Whether this property of MH41-7B is general for other class I or class II regulated transcripts remains to be established.

Our findings are reminiscent of results from a number of earlier studies suggesting that signals which can influence nuclear gene expression may originate from mitochondria. In *Neurospora crassa*, for example, Barath and Küntzel (2) observed that treatment of cells with chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, led to an increase in the synthesis of a number of nuclear-encoded proteins of the mitochondrial transcription and translation

apparatus. These investigators suggested that the mitochondrial genome encodes some repressor protein that acts in the nucleus to regulate gene expression. More recently, it was observed that when mitochondrial function in *N. crassa* was disrupted because of the *poky* mutation or when cells were grown on chloramphenicol, the mRNA level of *cyt-21*, a nuclear gene encoding a mitochondrial ribosomal protein, was substantially elevated (30). A similar observation has been reported in which chloramphenicol treatment of mouse erythroleukemia (Friend) cells blocked erythroid differentiation by blocking the induction of erythroid inducing factor II, which suggests that some mitochondrial product is a component of, or affects the expression of, this inducing factor (26).

These examples of the influence of the organelle and its genome on expression of nuclear genes are not restricted to mitochondria. There is evidence that in plants, expression of nuclear genes encoding some chloroplast as well as some nonchloroplast proteins is influenced by the differentiated state of the chloroplast (7) or by some mutational block in plastid development. It has been suggested that these effects, which are observed at the level of mRNA abundance, might be due to the presence of some chloroplast factor that affects nuclear gene expression.

NTS transcription. Our mapping data indicate that the most abundant NTS transcripts extend from a region of the rDNA repeat, close to the 5′ end of the 5S rRNA gene, downstream toward the 37S rRNA precursor to three prominent 3′ termini located between 1,780 and 1,880 bp from the 3′ end of the 26S rRNA gene. These mapping data are in reasonable agreement with 500- to 800-nt estimates for the sizes of the major NTS RNAs detected in Northern blots. Larger RNAs are apparent in these blots, and results obtained after long exposures of primer extension gels indicate that the 5′ ends of some NTS transcripts fall between the 5S rRNA gene and the enhancer element. The 3′ ends of the NTS transcripts do not correspond to any of the major precursor rRNA termination-processing sites that have been described (27). Therefore, it is unlikely that these abundant NTS transcripts are related directly to the synthesis of the 37S rRNA precursor.

There is now good evidence in some organisms that the NTS spacer region of the rDNA repeat contains important sequence elements for polymerase I transcription and is itself transcribed (51). In mice (20, 25) and *Xenopus laevis* (33, 35), for example, the NTS contains a termination site that overlaps the major polymerase I initiating site. This arrangement may function to pass on RNA polymerase I molecules directly from one transcription unit of the repeat to the next. In addition, the NTS region may function as a spacer promoter to load RNA polymerase I molecules into the rRNA precursor transcription unit (9, 35). In these examples, however, transcripts containing NTS sequences appear to be extremely unstable, so that their abundance is very low relative to the accumulation of precursor rRNA.

Unlike the situation in higher eucaryotes, the rDNA repeat in yeast cells contains the 5S rRNA gene embedded within the NTS in opposite orientation to the 18S, 5.8S, and 26S gene cluster (57). Such an arrangement could pose a problem if RNA polymerase I were to transcribe through the 5S gene. Kempers-Vennstra et al. (27) suggested that the NTS and 5S rRNA genes may be looped out of a transcriptionally active rDNA repeat, juxtaposing the polymerase I enhancer located just downstream of the 3′ end of the 26S rRNA gene, and the rRNA initiator elements. However, earlier in vitro experiments of Swanson et al. (54) suggested that the entire NTS of the yeast rDNA repeat is transcribed. Additional experiments will be required to determine whether the NTS in yeast cells has a function comparable to that of the NTS in higher eucaryotes.

The fact that the abundant NTS RNAs are polyadenylated, glucose repressible, and sensitive to inhibition by α-amanitin suggests that they are derived by polymerase II transcription. Although we have not defined the initiation region of the abundant NTS transcripts, it is noteworthy that there are three RNA polymerase II TATA box-like elements in the NTS at 139, 375, and 658 bp from the 3′ end of the 26S rRNA gene. Finally, the location of a consensus tripartite processing-polyadenylation sequence (59) near the 3′ termini of the major NTS transcripts is consistent with our results and with the presence of a polymerase II transcription unit within the rDNA repeat.

There have been a number of reports of RNA polymerase II transcription of sequences within the rDNA repeat. For example, Smale and Tjian (50) analyzed the transcription of herpes simplex virus thymidine kinase sequences fused to human RNA polymerase I promoter regions. They found that alterations in that promoter activated polymerase II transcription, resulting in some synthesis of thymidine kinase. Similar observations have been made with various rDNA constructs from *Drosophila melanogaster* (24). Recently, Santangelo et al. (49) identified a segment of NTS 1 in *S. cerevisiae* that could function in appropriate plasmid constructs as an RNA polymerase II promoter. Moreover, their studies indicate that divergent transcription occurs in vivo in that region of the rDNA repeat. One of the transcripts they identified, which is glucose repressible and is transcribed from the same strand as is the 37S precursor rRNA, may be the same as the regulated transcripts described here.

It is not clear how the switch to transcription of stable NTS RNAs occurs in certain respiration-deficient yeast strains. The available data show that the switch is linked in some way to the mitochondrial genotype and to a single nuclear locus, which together determine whether a given cell type expresses abundant NTS RNAs. In studies to be presented elsewhere (R. Docherty, H. Conrad-Webb, and R. A. Butow, manuscript in preparation), we have observed a unique regulation of rRNA synthesis in petite cells in addition to the well-established control of rRNA synthesis among all cell types that is tightly coupled to growth rate (29, 56). We are currently investigating the possibility that the NTS transcripts described here are related, either as cause or effect, to that unique regulation.

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