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Transcriptional Analysis of the *Saccharomyces cerevisiae* Mitochondrial *var1* Gene: Anomalous Hybridization of RNA from AT-Rich Regions

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A family of mitochondrial RNAs hybridizes specifically to the *var1* region on *Saccharomyces cerevisiae* mitochondrial DNA (Farrelly et al., J. Biol. Chem. 257:6581–6587, 1982). We constructed a fine-structure transcription map of this region by hybridizing DNA probes containing different portions of the *var1* region and some flanking sequences to mitochondrial RNAs isolated from *var1*-containing petites. We also report the nucleotide sequence of more than 1.2 kilobases of DNA flanking the *var1* gene. Our primary findings are: (i) The family of RNAs we detect with homology to *var1* DNA is colinear with the *var1* gene. Their direction of transcription is *oli1* to *cap*, as it is for most other mitochondrial genes. (ii) Extensive hybridization anomalies are present, most likely due to the high A-T (A-U) content of the hybridizing species and to the asymmetric distribution of their G-C residues. An important conclusion is that failure to detect transcripts from A-T–rich regions of the yeast mitochondrial genome by standard blot transfer hybridizations cannot be interpreted to mean that such sequences, which are commonly supposed to be spacer DNA, are noncoding or lack direct function in the expression of mitochondrial genes.

Different molecular weight forms of var1 polypeptide, a mitochondrial translation product associated with the small mitochondrial ribosomal subunit, are specified by alleles of the *var1* gene on *Saccharomyces cerevisiae* mitochondrial DNA (mtDNA) (6, 17, 22, 23, 26). An unusual feature of this gene is that it is about 90% A-T, with more than 30% of the G+C residues located within palindromic G-C–rich clusters (11). In this respect, the *var1* gene is more characteristic of nongenic *S. cerevisiae* mtDNA than of gene sequences.

In a previous report, we described four mitochondrial RNA (mtRNA) species (19S, 16S, 14S, and 13S) in wild-type cells, and in petite strains retaining *var1*, which hybridize specifically to *var1* DNA probes (8). Of particular interest is that, like var1 polypeptide, these RNAs also show strain-dependent size polymorphism: they vary in size in different strains in direct proportion to the size of the var1 protein, and the size differences among like species correspond roughly to the differences expected from our estimates of protein molecular weights. Of these four RNAs, we found that a 16S species is by far the most abundant in wild-type cells. Furthermore, in different mutant strains in which the amount of var1 polypeptide made was either substantially greater or less than in wild-type strains, the amount of 16S RNA also varied in the same direction. These observations led us to suggest that the 16S RNA is a good candidate for the mRNA for var1 protein.

Using hybridization procedures with RNA and DNA probes from both *var1*-containing petites and cloned *var1* sequences, the present work defined these RNAs as unique transcripts of the *var1* region. In addition, anomalies in the expected hybridization patterns were revealed which appear to be due, in part, to the high A-T content of the *var1* gene and the organization of G-C sequences within it. An important conclusion of these results is that failure to detect homology of RNAs to mtDNA sequences with high A+T content by conventional hybridization procedures cannot be interpreted per se to mean that such sequences are not transcribed or lack genetic function. This conclusion is particularly significant considering that about 50% of the yeast mitochondrial genome consists of long stretches of A-T–rich DNA interspersed with G-C–rich clusters (7, 11, 18), sequences which

generally have been considered but not proven to be devoid of a genetic role (3, 19).

MATERIALS AND METHODS

***S. cerevisiae* strains and growth conditions.** The relevant information on the strains used in this study is described in the Results. Strains were grown to mid-logarithmic phase in complete medium (1% yeast extract–1% peptone [Difco Laboratories]) containing either 2% galactose for wild-type or 2% glucose for petite cells.

Isolation and analysis of mitochondrial nucleic acids. MtDNA and mtRNA were isolated as previously described (8, 12). RNAs were separated by electrophoresis on 1.5% agarose–6 M urea gels (13). Transfer of RNA to diazobenzyloxymethyl (DBM)-paper was carried out essentially according to the method of Alwine et al. (1), with specific modifications as noted previously (8). MtDNAs were digested with restriction endonucleases purchased from New England Biolabs or Bethesda Research Laboratories and separated by electrophoresis on agarose or acrylamide vertical slab gels (8). Transfer of DNA to nitrocellulose (Schleicher & Schuell Co.) was according to Southern (21).

Conditions for labeling mtDNA and mtRNA, hybridization of the probes to RNA bound onto DBM-paper, or to DNA bound onto nitrocellulose, and visualization of labeled bands by autoradiography have been described previously (8). Briefly, all hybridizations were performed for 24 to 48 h at 42°C in 50% formamide–5× SSC (0.75 M NaCl–0.075 M sodium citrate)–50 mM $NaPO_4$ (pH 6.5)–0.2% sodium dodecyl sulfate (SDS)–1× Denhardt solution plus 1 mg of sonicated, denatured calf thymus DNA per ml. After hybridization, filters were washed five times for at least 30 min each in the same buffer at 42°C but without calf thymus DNA. Specific labeling procedures for the generation of end-labeled probes are described below and in the appropriate figure legends.

3′ End labeling and strand separation. About 5 μg of a 1-kilobase (kb) *Hin*dIII-*Eco*RI fragment, representing the entire *var1* sequence (and a few nucleotides of pBR322) cloned into the *Cla*I site of pBR322 (see below), were isolated by preparative electrophoresis and electroelution as described previously (27). After digestion with the *Mbo*I isoschizomer, *Sau*3AI, the resultant three fragments (see Fig. 6) were end labeled by fill-in with the Klenow fragment of DNA polymerase I (New England Biolabs) in a 50-μl reaction mixture containing: 1 μg of DNA, 67 mM Tris-hydrochloride (pH 7.4), 67 mM NaCl, 7 mM $MgCl_2$, and 50 μCi of [α-^{32}P]dGTP or [α-^{32}P]dATP (New England Nuclear Corp.). After 1 h of incubation at 20°C, the reaction was terminated with 2 μ of 0.5 M EDTA. The labeled bands were separated by electrophoresis through a 6% acrylamide gel (gel and running buffer: 0.1 M Tris-0.1 M boric acid-2 mM disodium EDTA). Labeled bands were identified by autoradiography, excised, and electroeluted. After ethanol precipitation, the individual fragments were electrophoretically strand separated and soaked out of the gel, as described by Maxam and Gilbert (16).

cDNA synthesis. MtRNAs were prepared from petite A17-10 and subjected to electrophoresis through preparative 1.5% agarose–6 M urea gels. The 16S band was electroeluted and purified by extraction with phenol-chloroform (1:1) and three ethanol precipitations from a 3 M ammonium acetate solution, followed by a final precipitation from 0.3 M sodium acetate.

The primer was a 46-base-pair (bp) *Sau*3AI-*Rsa*I fragment (see Fig. 1) prepared from pBR322-*var1* (see below). After the 5′ end was labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase, the primer was separated from free nucleotides and from higher-molecular-weight-labeled restriction fragments by electrophoresis through a 10% acrylamide gel and isolated by electroelution.

Hybridization of the primer to the 16S RNA template was performed as follows. The primer (2×10^6 cpm) plus 1 to 2 μg of 16S RNA were coprecipitated with ethanol, and the precipitate was dried and then dissolved in 20 μl of deionized formamide. After the mixture was heated at 68°C for 5 min and quickly cooled in ice, 5.6 μl of water and 3.2 μl of 10× hybridization salts (4 M NaCl, 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, pH 6.4], and 0.01 M EDTA) were added. Hybridization was carried out for a total of 3 h, decreasing temperature steps at one-half-hour intervals to yield 37, 30, 25, 20, 10, and 4°C. The nucleic acids were precipitated by the addition of 3 volumes of ethanol. The pellet was washed in 70% ethanol plus 1× reverse transcriptase salts (20 mM Tris-hydrochloride [pH 8.4]–10 mM NaCl–6 mM $MgCl_2$) and then dried.

cDNA was synthesized by dissolving the dried pellet in a 20-μl reaction mixture consisting of 1× reverse transcriptase salts, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 mM dithiothreitol, and 30 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.). After being incubated for 1 h at 42°C, the reaction was terminated by the addition of 1/10 volume of 1 M NaOH–10 mM EDTA and heating at 68°C for 15 min.

The cDNA was isolated by electrophoresis through a 6% acrylamide–8 M urea gel (gel and running buffer, 0.1 M Tris–0.1 M boric acid–2 mM EDTA). The 5′-end-labeled cDNA was identified by autoradiography, and the high-molecular-weight material was electroeluted. In zero-time control experiments and experiments in which RNA preparations were first treated with RNase, no labeled material migrating slower than the primer fragment was detected. The resultant cDNA preparations were chemically sequenced according to the procedure of Maxam and Gilbert (16).

Cloning of *var1* sequences. About 5 μg of mtDNA from A17-10 was digested to completion with *Hpa*II. Fragments were separated by electrophoresis on a 1% agarose gel and *Hpa*II fragment 2 (1 kb) was purified by electroelution, ligated into the *Cla*I site of pBR322, and cloned into HB101 by standard procedures. The cloned *Hpa*II-2 *var1* insert in pBR322 is shown in Fig. 1, and the clone is termed pBR322-*var1*.

Nucleotide sequence of *var1* flanking regions. For determination of flanking sequences, mtDNA was isolated from petite A17-10 as described previously (12). *Ava*II, *Hpa*II, and *Mbo*I digests were end labeled using the Klenow fragment of DNA polymerase I (New England Nuclear) as described above for probe labeling. The primary digests after being labeled were treated as follows: (i) the *Ava*II digest was secondarily digested with *Hin*cII to generate the asymmetrically labeled 241-bp fragment with coordinates −692 to

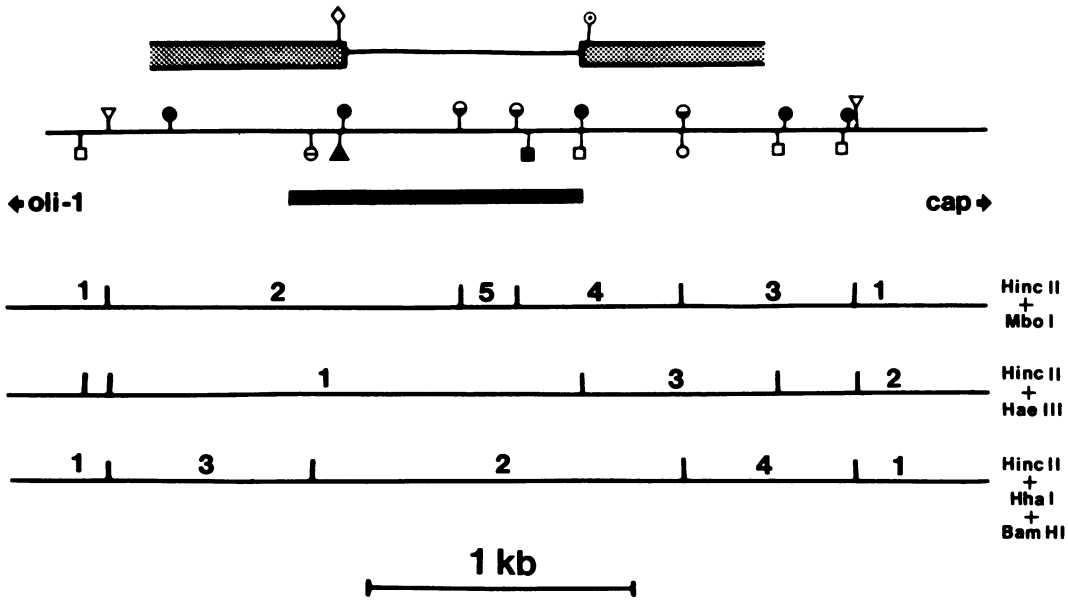

FIG. 1. Restriction endonuclease map of the *var1* region from petite A17-10 (*var1[40.0]*) mtDNA. Shown is the DNA segment of A17-10 mtDNA equivalent to *Hin*cII fragment 10 of wild-type mtDNA, and a small portion of the flanking sequences. The symbols on the thin horizontal line indicate the following restriction endonuclease sites: ▽, *Hin*cII; □, *Hae*III; ◒, *Mbo*I; ▲, *Hha*I; ○, *Bam*HI; ■, *Rsa*I; ●, *Hpa*II; ⊖, *Hin*fI. The thick horizontal line just below indicates the position of the open reading frame. The segment of *var1* DNA cloned into the *Cla*I site of pBR322 (*Hpa*II fragment 2 of A17-10 mtDNA) is shown in the top of the figure as the thin horizontal line flanked by pBR322 sequences (shaded areas). The *Eco*RI (⊙) and *Hin*dIII (◇) sites within pBR322 DNA are indicated. The map order of the various fragments generated by the different combinations of restriction endonuclease digests are shown in the bottom portion of the figure.

−451 (see Fig. 7). It was sequenced after electroelution from a 4% polyacrylamide gel. The *Ava*II fragment between coordinates −445 and +220 (not shown) was strand separated and sequenced in both directions. (ii) The *Hpa*II fragment between coordinates −773 (not shown) and −448 and between −447 to +213 (not shown) were strand separated and sequenced in both directions. The *Hpa*II fragment at +1,223 was digested with *Bam*HI and strand separated. (iii) The *Mbo*I fragment from +971 (not shown) to +1,503 was strand separated and sequenced in both directions, as was the *Mbo*I (*Bam*HI) fragment at +1,504 after being secondarily digested with *Hin*cII at a site 3′ to the region shown in Fig. 7.

Doubly labeled fragments were strand separated by electrophoresis through previously described 4% or 6% polyacrylamide gels before electroelution (16). Sequencing reactions were those of Maxam and Gilbert (16) and Rubin and Schmid (20), modified as previously noted (11).

RESULTS

Fine-structure transcriptional analysis of the *var1* region. To locate more precisely the regions of homology of the *var1* RNAs to the *var1* region, mtDNA from a petite strain (A17-10), was digested with various restriction endonucleases, and isolated fragments were used individually as hybridization probes against mtRNA in a Northern blot analysis. Strain A17-10 is arranged as a 5.3-kb head-to-tail tandem repeat containing the entire *var1* gene and about 4 kb of flanking sequences (26). The mtRNAs analyzed were also isolated from petite A17-10. We have previously shown that this strain, as well as other petites containing the entire *var1* gene, make RNA species with homology to *var1* which are indistinguishable from those found in wild-type strains (8).

Three combinations of restriction digests were used to furnish hybridization probes covering the *var1* region: *Hin*cII + *Mbo*I, *Hin*cII + *Hae*III, and *Hin*cII + *Hha*I + *Bam*HI. The map order of the above restriction fragments in the relevant part of A17-10 mtDNA is shown in Fig. 1. For *Hin*cII + *Mbo*I and the *Hin*cII + *Hha*I + *Bam*HI digest, the largest fragment is the junction piece of the tandem repeat; all of the others correspond to wild-type sequences within *Hin*cII fragment 10. For the *Hin*cII + *Hae*III digest, only the three largest fragments were isolated of the nine produced. The appropriate restriction fragments from all of these digests were purified by two successive rounds of elec-

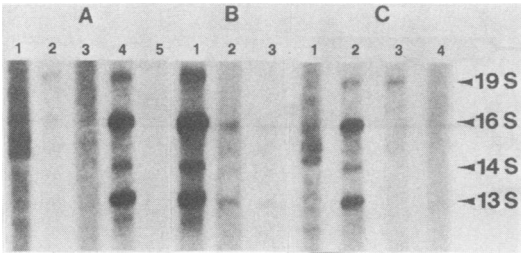

FIG. 2. Northern blot hybridization of mtRNAs from petite A17-10. MtRNAs were isolated from A17-10, separated on 1.5% agarose–6 M urea gels and blotted onto DBM paper as described previously (8). Identical strips were cut and hybridized individually with nick-translated DNA probes. The numbers above each lane refer to the specific fragment used as a probe, as shown in Fig. 1 for the three digests: (A) *Hin*cII + *Mbo*I; (B) *Hin*cII + *Hae*III; and (C) *Hin*cII + *Hha*I + *Bam*HI. The positions of the major *var1* RNAs are indicated on the right.

trophoresis through agarose gels and labeled with ^{32}P by nick translation.

The results of hybridization of these different probes to DBM blots of mtRNA from petite A17-10 are shown in Fig. 2. The 16S, 14S, and 13S RNAs show strong hybridization to only one fragment in each of the digests: *Hin*cII + *Mbo*I, fragment 4; *Hin*cII + *Hae*III, fragment 1; and *Hin*cII + *Hha*I + *Bam*HI, fragment 2. The 19S species also hybridizes to these fragments as well as to fragment 3 of the *Hin*cII + *Hha*I + *Bam*HI digest. The 16S RNA shows somewhat weaker hybridization to *Hin*cII + *Mbo*I, fragment 2.

The above strong hybridizations have in common only about a 250-bp segment of the *var1* region bounded by an *Mbo*I and a *Hae*III site. Since we have previously determined (8) by electrophoresis on methylmercury-agarose gels that the 19S and 16S RNAs are about 2,600 and 2,000 nucleotides long, respectively, we obviously cannot account for the full length of these RNA species as transcripts of *var1* DNA, based on these Northern blot hybridizations. Thus, we conclude either that complementary sequences do not form stable hybrids under the hybridization conditions employed, or that these RNAs contain sequences not derived from *var1* DNA. Investigation of these possibilities is described below.

In addition to the *var1* RNAs, a number of additional discrete RNA species are observed in these blots. Since these RNAs hybridize predominantly to the junction fragments of the repeat unit and are absent from wild-type strains, they are most likely transcripts which include sequences from the junction piece of the petite mtDNA repeat unit.

Analysis of cDNA synthesized from the 16S RNA. Figure 2 (lane A5) shows particularly clearly the complete lack of hybridization of any of the RNA species to the small *Mbo*I-bounded fragment which, based on DNA sequencing, is present in the *var1* coding region. One interpretation of the hybridization results is that they faithfully reflect a splicing event such that the sequences in the small *Mbo*I fragment do not appear in the *var1* message. This interpretation was tested explicitly by synthesizing and sequencing cDNA primed on purified 16S RNA so as to extend into the sequences, if present, encoded by the *Mbo*I-bounded fragment. To do this we used a 5′-end-labeled 46-bp *Mbo*I-*Rsa*I fragment which abuts the 3′ end of *Hin*cII + *Mbo*I, fragment 5, as a primer to synthesize cDNA from a purified 16S RNA template. Figure 3 shows a portion of the nucleotide sequence of this cDNA. The sequence shown is colinear with the DNA sequence within *Hin*cII + *Mbo*I,

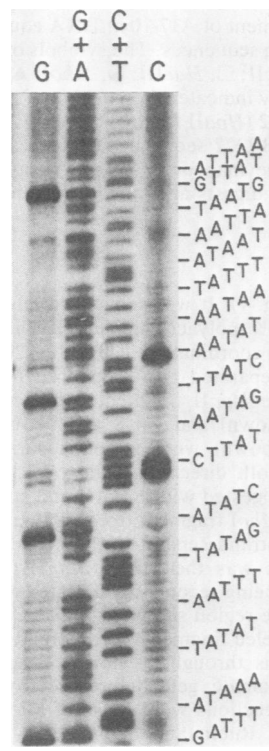

FIG. 3. Nucleotide sequence of cDNA from a 16S RNA template. Shown is the autoradiogram of a sequencing gel of a portion of a cDNA copy of 16S RNA. The separate base-specific reactions are indicated at the top of each lane. The derived sequence, starting at nucleotide number 57 from the 5′ end of the *Sau*3AI-*Rsa*I primer, is identical to the corresponding DNA sequence of the *Mbo*I-*Mbo*I fragment from A17-10 as previously reported by us (11).

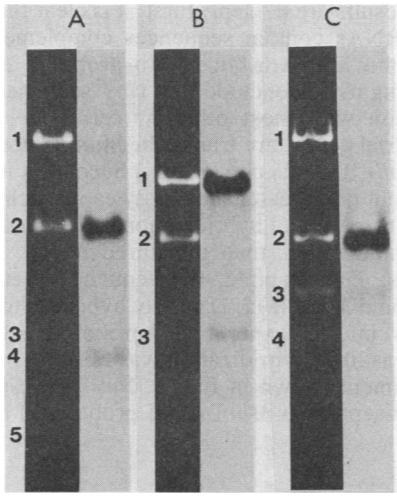

FIG. 4. Hybridization of labeled 16S RNA to restriction fragments from A17-10 mtDNA. Isolated 16S RNA, prepared from A17-10, was digested mildly with alkali and 5′ end labeled with polynucleotide kinase, as described previously (8). The labeled RNA was used to probe a Southern blot of restriction digests of A17-10 mtDNA electrophoretically separated through a 1% agarose gel. Shown are the ethidium bromide-stained gel patterns of the restriction digests next to autoradiograms of the Southern blot. Lanes A: *Hin*cII + *Mbo*I; lanes B: *Hae*III; lanes C: *Hin*cII + *Hha*I + *Bam*HI. To the left of each lane are indicated the fragment numbers which correspond to the same probes as used in the Northern blot analysis shown in Fig. 2.

fragment 5, previously determined by us on A17-10 mtDNA (11). Thus, our result shows that the failure of this segment of the *varl* region to hybridize to the 16S RNA is not due to the absence of those sequences in the transcript, but rather implies the inability to form stable hybrids under the conditions used to assay homology.

Detailed studies with 16S mtRNA. Additional hybridization studies were carried out with 16S RNA purified as a hybridization probe. This RNA species can be obtained greater than 90% pure by oligodeoxythymidylate-cellulose chromatography and electroelution from agarose-urea gels (8). The purified 16S RNA was treated with mild alkali to generate fragments and thus eliminate most secondary structure. The fragments were then 5′ end labeled with ^{32}P and used as hybridization probes against a Southern blot of DNA restriction fragments from A17-10.

The results shown in Fig. 4 clearly reveal regions of homology not detected by the previous Northern blot analysis (Fig. 2). For example, the labeled 16S RNA gives a strong hybridization signal to *Hin*cII + *Mbo*I, fragment 2, whereas in the Northern blot of Fig. 2, this fragment hybridizes only weakly to the 16S RNA. A summary of those fragments which show strong hybridization to the labeled 16S RNA probe is presented in Fig. 5 (experiment 2). From the pattern of hybridization, the portion of the *varl* region which shows sequence homolo-

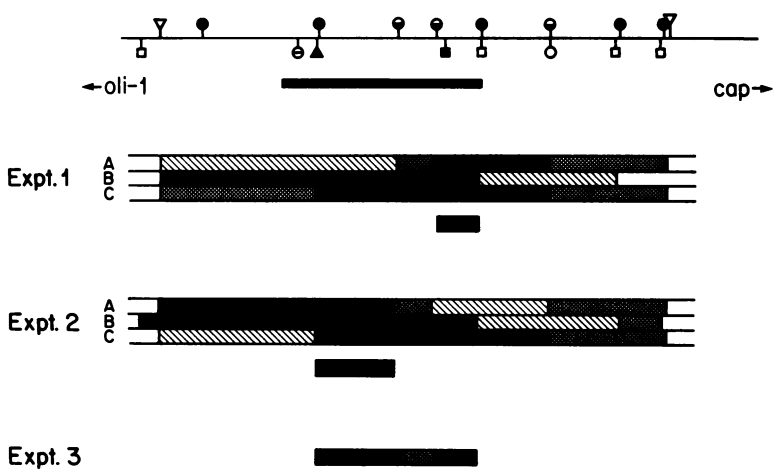

FIG. 5. Summary of hybridization results of the 16S RNA to the *varl* region. Diagrammed is the homology detected between the 16S RNA and restriction fragments from the *varl* region. The restriction endonuclease map, open reading frame, and symbols of the *varl* region are reproduced from Fig. 1. Experiment (Expt.) 1 summarizes the results of Northern blots of A17-10 mtRNAs probed with double-stranded DNAs (from Fig. 2); experiment 2 summarizes the results of Southern blots of restriction fragments of A17-10 mtDNA probed with purified 16S RNA (from Fig. 4); experiment 3 diagrams the homology of single-stranded DNA probes to Northern blots of A17-10 mtRNAs (from Fig. 6). A strong hybridization signal is indicated by solid bars, and a weak hybridization signal is indicated by cross-hatched bars. Stippled bars represent no detectable hybridization. For experiments 1 and 2, a transcription map, deduced from the pattern of hybridization, is also diagrammed. A, B, and C indicate the restriction digests used to generate either probes (Expt. 1) or fragments blotted and probed with labeled 16S RNA (Expt. 2).

gy to the 16S RNA is now extended to include sequences between the *Hha*I and *Mbo*I sites (Fig. 1).

The failure to obtain identical results in the complementary experiments involving *Hinc*II + *Mbo*I, fragment 2, emphasizes the caution needed in interpreting yeast mitochondrial transcript mapping. Since ribouridine-deoxyadenylate (rU-dA) base pairs are uniquely unstable in duplexes (15) (see below), we suggest that the reannealing of the denatured duplex probe in the Northern blot analysis (Fig. 2) competed significantly with the desired RNA-DNA hybridization. Control experiments (not shown) demonstrated that the DNA probes used were available for hybridization. In these control experiments, DNA blots identical to that depicted in Fig. 4 were hybridized with nick-translated *var1* DNA probes obtained from the pBR322-*var1* clone (Fig. 1). All restriction fragments of A17-10 mtDNA, including the *Mbo*I-*Mbo*I piece, which are contained within the cloned *var1* DNA, hybridized well with the probe.

In addition to the one strongly hybridizing band in each lane of Fig. 4, each lane shows at least one weakly hybridizing band. The positions of these weakly hybridizing bands within the *var1* region is also shown in Fig. 5 (experiment 2). Interestingly, the DNA segment between the *Mbo*I and *Hae*III sites, which was the only region showing strong hybridization to the 16S RNA in the Northern blot analysis, shows only a weak signal when the DNA is probed with labeled RNA, a result which is unexplained. A weak signal intensity is also seen for those fragments which did not hybridize in the Northern blot analysis, namely, *Hinc*II + *Hha*I + *Bam*HI, fragment 3, and *Hinc*II + *Hae*III, fragment 3. Finally, in neither of these hybridization experiments was there any hybridization of the 16S RNA to the 250-bp DNA sequence bounded by the *Mbo*I sites (*Hinc*II + *Mbo*I, fragment 5).

Analysis with single stranded DNA probes. Kinetic considerations predict that separated, single-stranded hybridization probes offer a greater degree of sensitivity than unseparated probes. Thus, we examined the hybridization pattern of defined, single-stranded *var1* probes to *var1* RNAs and, at the same time, determined the direction of transcription of *var1* sequences. Single-stranded probes were obtained from the cloned *var1* DNA and used in Northern blot analysis. A *Hin*dIII + *Eco*RI fragment from pBR322-*var1* (Fig. 1) was digested with *Sau*3AI, and the resultant three fragments were labeled at their 3′ ends as described above.

Figure 6A depicts each of the single-stranded probes used, and Fig. 6B shows the hybridization of these probes to a DBM blot of mtRNAs from wild-type strain ID41-6/161 (*var1[40.0]*). Two results are evident: First, it is clear that the *var1* RNAs contain sequences complementary to probes 2 and 6 but not to probes 1 and 5, allowing us to conclude that *var1* sequences, in common with most other *S. cerevisiae* mitochondrial genes, are transcribed in the direction from *oli1* towards *cap* (Fig. 1). Second, it is also apparent that greater sensitivity can be achieved in a Northern blot hybridization by using strand-separated rather than denatured duplex DNA probes. For example, the sequences between *Hha*I and *Mbo*I (Fig. 1) clearly hybridize to *var1* RNAs in this experiment (probe 1, Fig. 6), whereas little hybridization was observed in the experiment shown in Fig. 2. Nevertheless, the strand-separated *Mbo*I-*Mbo*I probes still fail to

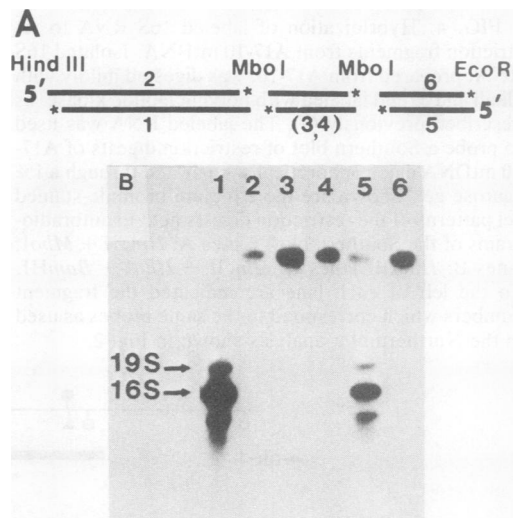

FIG. 6. Hybridization of *var1* RNAs with strand-separated DNA probes. (A) The cloned *var1* DNA was isolated from pBR322-*var1* as a *Hin*dIII + *Eco*RI restriction fragment (Fig. 1), digested with *Sau*3AI, and 3′ end labeled as described in the text. After isolation of each labeled fragment by electrophoresis, the fragments were denatured and electrophoretically strand separated. The individual single-stranded probes are numbered 1 through 6, and labeled 3′ ends are indicated by asterisks. Each probe was used in the analogously numbered lane in (B). For the strand-separated *Mbo*I-*Mbo*I fragment, the slower migrating strand is designated probe 3 and the complementary faster migrating strand is designated probe 4. (B) mtRNAs from strain ID41-6/161 (respiratory competent cells, [ρ^+], *var1[40.0]*) were subjected to electrophoresis through a 1.5% agarose–6 M urea gel and transferred onto DBM paper. The Northern blots were hybridized with the following amounts of the probes described in (A): lane 1, 26,000 cpm of probe 1; lane 2, 11,000 cpm of probe 2; lane 3, 61,000 cpm of probe 3; lane 4, 57,000 cpm of probe 4; lane 5, 93,000 cpm of probe 5; lane 6, 246,000 cpm of probe 6. The top band seen in lanes 2 to 6 is mtDNA.

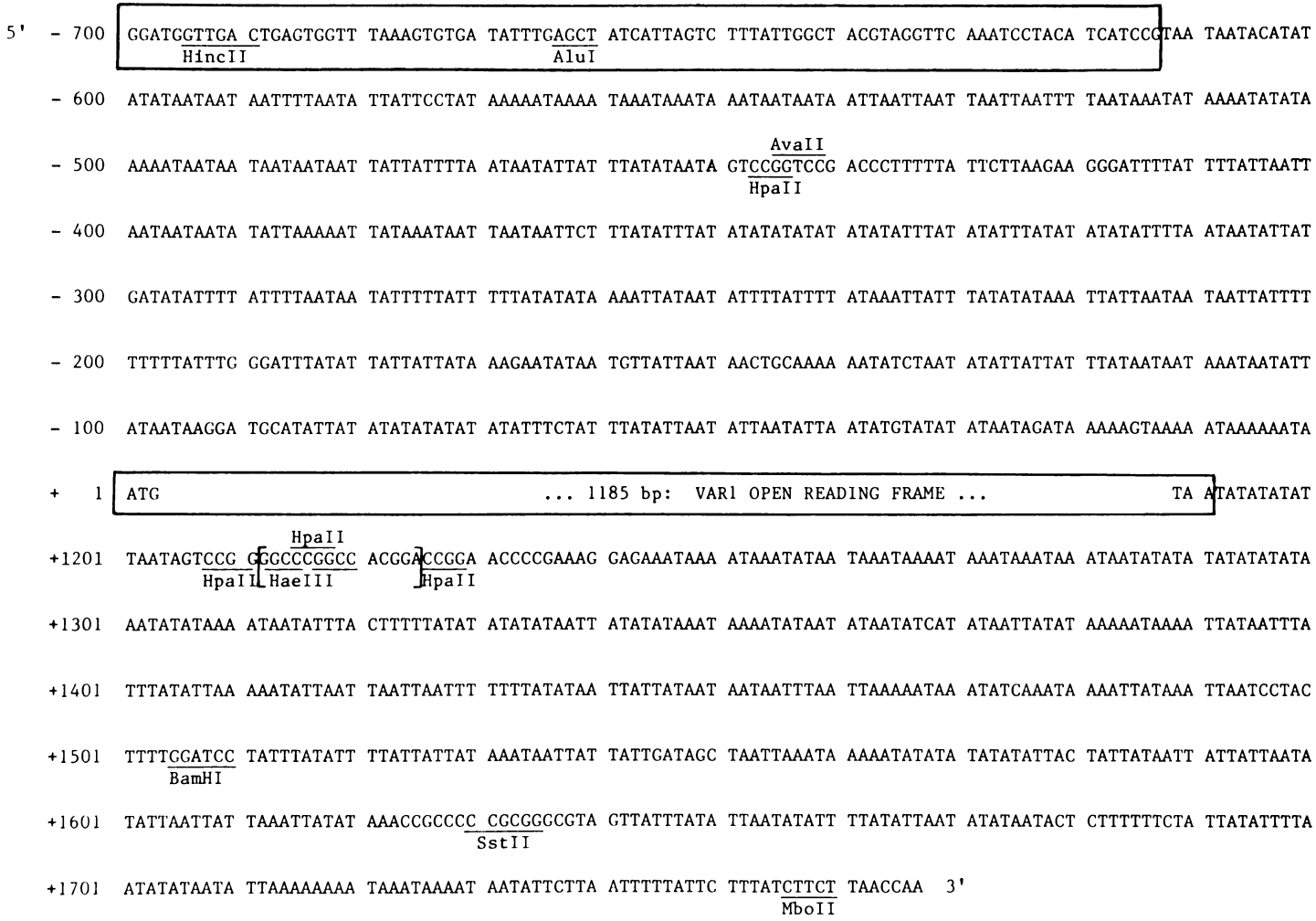

FIG. 7. Nucleotide sequence of regions flanking the *varl* gene. Shown is the nucleotide sequence of the nontranscribed strand from a petite strain (A17-10) carrying the *varl[40.0]* allele. The bracketed sequence starting at +1,212 is taken from the sequence of the *varl[42.0]* allele reported by Tzagoloff et al. (24) and has not yet been confirmed by us. We are not able to confirm the *Ava*II site (GGACC) which starts within that bracketed region. All other restriction sites indicated are consistent with their known locations. The boxed region between nucleotides −700 to −614 encodes the $tRNA^{Ser II}$ gene. The A at −451 is uncertain.

hybridize to the RNAs (Fig. 6, lanes 3 and 4), whereas in control experiments, the individual probes hybridize well to the mtDNA present on the blot.

Sequence of DNA segments flanking the *var1* gene. The DNA sequence of the *var1* region was examined to see whether sequence considerations could offer some insight into the lack of hybridization of some restriction fragments to the *var1* RNAs. We recently reported the nucleotide sequence of the coding region of the *var1[40.0]* allele studied in the present work (11), and here we present the sequence of the flanking regions of that allele (Fig. 7). Portions of these flanking sequences have also been reported for a *var1*-containing petite strain (DS401) which retains the *var1[42.0]* allele (24).

Analysis of the entire 2.6-kb region failed to reveal significant hairpin homology which could affect the single-stranded nature of any of the probes used; the longest hairpin contained 21 bp but was wholly lacking in G-C residues.

We also evaluated the content of G+C and U residues of defined portions of the *var1* RNAs versus the hybridization results of those portions to the homologous DNA fragments (Table 1). The hybridization signal obtained for each region tabulated does not correlate in a simple way with either parameter. Additional factors (see below), thus, appear to play an important role in the formation of detectable RNA-DNA hybrids in this system.

DISCUSSION

Our DNA sequencing of the genetically defined *var1* region has shown that it contains an open reading frame of sufficient length to encode the entire var1 protein (11). Furthermore, the predicted amino acid composition of a protein translated from that sequence agrees well with that experimentally determined from the purified var1 protein (11). Preliminary transcription mapping indicated, however, that only a small portion (approximately 250 bp) of the *var1* region was represented in a family of transcripts unique to *var1* (4). With the documentation in this paper of the extensive hybridization anomalies between the *var1* RNAs and DNA probes, we can now reconcile this apparent discrepancy. A very important implication of these results is that the failure to detect stable transcripts from particular regions of the *S. cerevisiae* mitochondrial genome does not guarantee that coding or regulatory functions are absent.

The transcript mapping data from the strand-separated probes (Fig. 6) and the sequencing of 16S RNA cDNAs (Fig. 5) reveal that the *var1* gene is transcribed in the same direction as all the other genes encoding known *S. cerevisiae* mitochondrial polypeptides. Using the genetic markers flanking the *var1* gene as reference points, this direction is *oli1* towards *cap*. This conclusion is entirely consistent with the DNA sequencing results which revealed an open reading frame in the *var1* region only if it were transcribed in that direction (11). Our cDNA sequencing also confirms the orientation and sequence of the *Mbo*I-*Mbo*I restriction fragment in the *var1* region (Fig. 1), determined from DNA sequencing of the *var1[40.0]* allele. This fragment had been incorrectly assigned the inverted orientation on the basis of DNA sequencing of the *var1[42.0]* allele in strain DS401 (24).

In part, we can understand the anomalous RNA-DNA hybridization patterns on the basis of the base composition and nucleotide sequence of the *var1* region. At least three interacting factors seem to determine whether stable RNA-DNA hybrids are detected. First, the greater the G+C content of the hybridizing reactants is, the higher is the stability of possible hybrids. This is clearly evident upon comparing the hybridization to the *var1* RNAs of the *Mbo*I-*Mbo*I fragment (5.8% G+C) versus the *Mbo*I-*Hae*III fragment (14.5% G+C) (Fig. 3 and Table 1). The former failed to show hybridization by all methods, whereas the latter was positive in all tests. We have also performed the Northern blot hybridizations at reduced stringency. In our standard conditions (50% formamide–5× SSC) the calculated melting temperature (T_m) for DNA fragments of 5% G+C composition is 52°C (5). Thus, in the case of the *Mbo*I-*Mbo*I restriction fragment, for example, we hybridized the fragments at T_m − 10°C. When we reduced the stringency to T_m − 17°C (hybridization temperature of 35°C), the specific signal from using that DNA fragment as a probe in the Northern blots did not increase; merely, the intensity of the background signal became more prominent. This result was also seen using the other DNA restriction fragments from the *var1* region as probes. Similar observations have been reported by Locker and Rabinowitz for hybridization of 21S rRNA precursors to mtDNA restriction fragments (14). Thus, the failure to detect specific DNA-RNA hybridization is not simply a consequence of employing too stringent hybridization conditions for DNA of low G+C base composition.

Second, counteracting the hybrid stability conferred by the G+C residues is the U content of the RNA. Martin and Tinoco have demonstrated the unique instability of rU-dA duplexes compared to duplexes formed from rA-dT or dA-dT base pairs (15). Thus, potential rU-dA base pairs in the *var1* region not only do not contribute to the stability of hybrids, but probably destabilize short regions of homology under

TABLE 1. Hybridization of *var1* RNAs to defined *var1* DNA segments as a function of RNA base composition

Fragment	Length (Nucleotides)	% G+C	% U	Hybridization[a]				
				Northern				Southern 16S RNA probe
				Double-stranded probe		Single-stranded probe		
				19S	16S	19S	16S	
−613 → *Hha*I	812	7.0	44	+	−	NT	NT	±
−163 → *Hha*I	362	9.4	36	−	−	NT	NT	±
*Hha*I → *Mbo*I	551	11.3	39	−	−	+	+	+
*Mbo*I → *Mbo*I	223	5.8	44	−	−	−	−	−
*Mbo*I → *Hae*III	241	14.5	39	+	+	+	+	±
*Hae*III → *Bam*HI	287	8.4	37	−	−	NT	NT	±
*Bam*HI → +1737	232	11.6	46	−	−	NT	NT	−

[a] The hybridization results presented in Fig. 1, 2, and 6 are correlated with the base composition of the portions of the *var1* region being probed. *var1* segments tested are listed in order (top to bottom), starting from the 5′ end of the largest *var1* RNA (19S), in sections bordered by restriction sites, through to the coterminal 3′ end of the 16S and 19S RNAs. The mapping of the 5′ and 3′ ends of these RNAs was determined by S1 nuclease protection analysis (data to be presented elsewhere), and the ends are indicated with reference to the sequence given in Fig. 7. For those restriction fragment probes which extend beyond the ends of the RNA (*Hinc*II-*Hha*I for the 5′ ends and *Bam*HI-*Hinc*II for the coterminal 3′ end), only that portion of the DNA homologous to the RNA is listed. The base composition of each section given is that of the RNA. The hybridization results are organized according to the procedure used as indicated above. The hybridization signal is scored as strong (+), weak (±), or absent (−); those sections not tested by a particular method are indicated by NT.

our hybridization conditions. This may be the primary reason why we can easily detect DNA-DNA hybridization in Southern blots with the same probes that fail to give DNA-RNA hybridization in Northern blots (Fig. 6).

Third, the DNA sequence of the *var1* region shows that a large proportion of the G+C residues fall into clusters which can adopt secondary structures. Such structures, formed by unimolecular reactions, would be kinetically favored over bimolecular hybrids and thus remove those G+C residues from participation in RNA-DNA hybridization. We have obtained evidence for the presence of such structures in the *var1* RNAs from the results of our cDNA syntheses using the 16S RNA as a template (H. P. Zassenhaus and R. A. Butow, unpublished data). The presence of short, contiguous stretches of G+C residues may be important, nevertheless, as nucleating sites for hybridization, particularly if such stretches do not adopt secondary structures. This may account for the observation that a *Hinc*II-*Hha*I probe hybridized to the 19S but not to the 16S RNA. The G+C or U content of the portion of these two RNAs homologous to that restriction fragment is not very different (Table 1). The 19S RNA, however, which extends a few hundred bases 5′ of the 16S RNA (data to be presented elsewhere) contains a nonpalindromic stretch of 15 bases, 12 of them G+C, which may kinetically promote hybridization (Fig. 7, residues −437 to −451). A similar result has been reported by Grivell et al. (9) in their transcript mapping of *cob-box* RNAs. The *cob-box* mRNA contains a long A+U-rich leader which could only be driven into a hybrid with homologous restriction fragments by using probes which extended into the relatively G+C-rich upstream portions.

Taken together, our analyses of the *var1* RNAs by hybridization methods and cDNA sequencing, and the previously described characterization and sizing of those RNAs (8), suggest that the larger RNAs include the entire *var1* gene plus extensive amounts of flanking sequences. Indeed, the 19S RNA, a 2,600-base transcript, seems to extend several hundred bases beyond the 5′ and 3′ borders of the open reading frame. Such long flanking portions to the coding sequences in stable gene transcripts are characteristic of the *S. cerevisiae* mitochondrial system (10) and are in marked contrast to animal mitochondria in which the stable gene transcripts include at most a few bases of noncoding sequences (2, 25). Unique to the *var1* gene is the finding that the flanking regions are not obviously different from the coding portion in their sequence organization and base composition (Table 1; also cf. Fig. 7 with Fig. 3 of reference 11). This similarity stems primarily from the unusually high A+T content (about 90%) of the *var1* gene when compared to other yeast mitochondrial protein genes.

The importance of our analysis of the long *var1* flanking sequences stems from the genetic demonstration that these flanking sequences are not without function; at least two *var1* mutations have been described which apparently map to

those segments (28). The latter observation, and the difficulty in detecting RNAs transcribed from DNA of such a base composition, suggest that the spacer function previously ascribed to such sequences needs reappraisal. Since nearly 50% of the yeast mitochondrial genome is comprised of long stretches of very A+T-rich DNA interspersed by short G+C-rich clusters (3, 18), the information content of this genome may not only be higher than heretofore thought possible, but perhaps also utilized in novel ways.

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