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Effect of point mutations on *in vitro* transcription from the promoter for the large ribosomal RNA gene of yeast mitochondria

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ABSTRACT

Initiation of transcription on mitochondrial DNA of Saccharomyces cerevisiae was studied in an in vitro system with a mtRNA polymerase fraction reconstituted from separately purified components and with DNA templates containing the promoter of the gene coding for large rRNA. The effect of various point mutations in this promoter region was quantitated in assays containing a wildtype promoter in equimolar amount as internal control. Despite the strong conservation around the position at which RNA initiation occurs (ATATAAGTApuTA, initiation nucleotide underlined), none of the single point mutations abolished transcription-initiation completely. Some reduce the efficiency of initiation to 10-20% compared to the wild type promoter, while others have a much less pronounced effect. A change of the A at position +4 into a G even results in a promoter up mutation. Remarkably, alteration of the A at position +1 into a G or a T affects the efficiency of initiation only slightly and initiation is maintained at the same position.

INTRODUCTION

Mitochondria of Saccharomyces cerevisiae contain a circular genome of about 80 kb which encodes rRNAs, tRNAs, an RNA involved in tRNA processing and proteins that function in the respiratory chain complexes or in RNA processing and splicing (reviewed in 1). Analysis of the multiple sites on the genome where transcription initiation takes place led to the discovery of a highly conserved sequence of nine nucleotides ($\frac{A}{T}$TATAAGTA) (2,3). Transcription starts at the last A (position +1, see figure 1) of this nonanucleotide motif. Comparison of all strong promoters also suggests a role for the motif puTA generally found at position +2, +3 and +4 (3). The development of an in vitro transcription system using partially purified mtRNA polymerase (4,5) made it possible to test the sequence requirements for specific transcription initiation. Bal-31 deletion studies of the small and large rRNA promoters confirmed that the nonanucleotide sequence is an important element of the mitochondrial promoter (6,7). When a short synthetic promoter homologous to nt -13

to +4 of the small rRNA promoter was cloned into a bacterial vector it was found to support specific transcription initiation in vitro. All substitutions of the -2 G nucleotide in this synthetic promoter completely abolished transcription, whereas substitutions of purine at position +2 and, to a lesser extent, of the T at position +3 decreased the activity of the promoter (8,9). An inhibitory role of a T at position +2 was also suggested by in vitro analysis of a number of mitochondrial tRNA promoters and a weak promoter downstream of the strong promoter belonging to the ATPase subunit 9 gene (8,10). Furthermore, two single point mutations (-6 C or -2 T) introduced by in vitro site-directed mutagenesis into the promoter of the large rRNA gene each drastically reduce in vitro transcription (7).

All in vitro transcription experiments described above were carried out with partially purified preparations of mtRNA polymerase, which was once thought to consist of only one protein component (5). Further purification and characterization of the polymerase has however demonstrated that it consists of at least two different functional components which can be separated by chromatographic methods (7,11). One component performs RNA elongation, but is unable to initiate correctly on mitochondrial promoters. The other component is transcriptionally inactive but it causes the first component to start transcription at the +1 site in the promoter. Because of the similarity between this functional subdivision and that of E.coli RNA polymerase (12) we call the RNA elongating component core polymerase, and the other component specificity factor. A shortage of specificity factor in in vitro transcription reactions can obscure the interpretation of the effect of introduced point mutations (7,13). Since we now have at our disposal extensively purified and separated components of mtRNA polymerase (7,14; Schinkel et al., submitted) we can analyze the effect of mutations in the mitochondrial promoter in a more defined manner than has been possible so far. This article describes the construction of a number of point mutations in the large rRNA promoter, and a quantitation of the effects of these mutations on transcription efficiency in an in vitro transcription system using mtRNA polymerase reconstituted from extensively purified core and specificity factor fractions.

MATERIALS AND METHODS

Recombinant DNA methods. Restriction enzymes were obtained from Boehringer Mannheim and New England Biolabs, T4 DNA ligase was from New England Biolabs and DNA polymerase I, Klenow fragment from Boehringer Mannheim. Sequence reactions were carried out using the dideoxy chain termination method (22). Recombinant DNA techniques were carried out according to standard procedures (15).

Recombinant plasmids. Mc21A, Mc21A-48/+46, Mc21B-211/+46, Mc21A-T/G^{-101} pU21H and pB21H have been described before (7,16). pU21-T/G^{-101} was constructed by cloning the BamHI-insert of Mc21A-T/G^{-101} into the BamHI site of pUR222. Mc21A-211/+46 was obtained by cloning the insert of Mc21B-211/+46 into M13mp19. pB21H/mutant or wild type combination clones were obtained by cloning the EcoRI-HindII inserts of Mc21A-48/+46 into EcoRI- and EcoRV-digested pB21H. Mc21A clones containing -6 C, -2 T or +4 G mutations were digested with AhaIII (cutting at position +46) and after ligation of a SalI linker inserts containing mtDNA from nt -211 to +46 were recloned in EcoRI-EcoRV-digested pB21H. pB21H/20A1 was obtained by ligating the EcoRI-insert of M-20A1 (2) into EcoRI-digested pB21H.

Construction of point mutations

Variations of the synthetic 17-mer oligonucleotide ATATAAGTAGTAAAAAG corresponding to nt -8 to +9 of the large rRNA promoter were used to mutagenize Mc21A48/+46 or Mc21A (7). Sets used in each mutagenesis procedure were Tab 1 (-2 A or C), Tab 2 (-1 G, A or C), Tab 3 (+1 G, T or C), Tab 4 (+2 A, T or C), Tab 5 (+3 G, A or C) or Tab 6 (+4 G, T or C). The mutagenesis procedure was carried out in principle as described (17,18) using a double priming method with a mutagenic primer and the 18-mer TGTAAAACGACGGCCAGT hybridizing just upstream of the insert on single stranded Mc21A-48/+46. After filling in with Klenow fragment of DNA polymerase I and ligation, the insert was isolated by digestion with EcoRI and HindIII and recloned into EcoRI- and HindIII-digested M13mp18 or M13mp19. Mutants were selected by direct sequence determination on ssDNA of resulting clones. Construction of the -6C and -2T mutants has been described before (7).

mtRNA polymerase fractions

Core RNA polymerase was purified from 3 kg of a S.cerevisiae strain ("Koningsgist", kindly provided by Gist-Brocades) as described (14) up to the glycerol gradient step. The specific core activity of the C2 fraction used in promoter analysis is 71 units per mg protein. Assuming the core component to be a 145 kDa protein (14) we estimate that it represents 5-10% of protein in the C2-fraction. The specificity factor of mtRNA polymerase was purified from the same starting material as described by us (Schinkel et al., submitted) up to the glycerol gradient step. The specific factor activity of the F8 fraction used in promoter analysis is about 1200 units per mg protein. Assuming the specificity factor to be a 43 kDa protein this fraction consists for about 50% of specificity factor. One unit of core polymerase activity is defined as the amount of enzyme which incorporates 1 nmole of UTP in a 155 nt run-off transcript synthesized from HindIII-digested pU21H in the presence of a saturating amount of specificity factor in a standard run-off assay (see below). One unit of specificity factor activity is defined as the amount of enzyme which stimulates incorporation of 1 nmole of UTP in specific 155 nt run-off product in the presence of 5 x 10^{-3} unit core polymerase under standard conditions.

Transcription reactions

Preparation of digested template for run-off transcription was carried out as described (7). For short run-offs of wild type and +1 T mutant SalI-BglII-inserts (run-off length 52 nt) or HindII-BglII-inserts (run-off length 50 nt) of Mc21A-48/+46 clones were isolated by agarose gel electrophoresis (15) and transcribed at about 0.7 pmol per reaction. For the +1 G mutant a SalI-AvaII-insert was isolated from Mc21B-48/+46. Transcription reactions were carried out in 25 µl at 25°C for 20 minutes as described (4). Reaction mix-

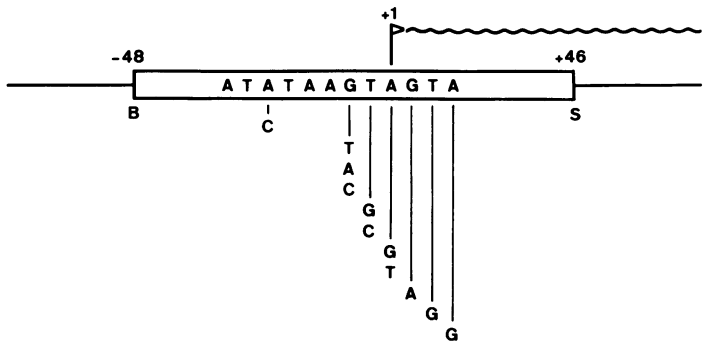

Figure 1: Schematic representation of the mtDNA insert containing the large rRNA promoter region in Mc21A-48/+46 and listing of single point mutations introduced by in vitro site-directed mutagenesis.

The sequence of the 12 nucleotides conserved in strong promoters is given as it occurs in the large rRNA promoter. The flag indicates the initiation nucleotide (+1) for mtRNA polymerase, the wavy line RNA. The boxed region represents mtDNA sequence, the line vector sequences. B = BamHI, S = SalI. Mutations -6 C, -2 A and +4 G are present on -211/+46 mtDNA fragments, all other mutations on -48/+46 mtDNA fragments. The figure is not drawn to scale.

tures were at 40 mM KCl and contained (unless indicated otherwise) 1 µg of template DNA, 7.10^{-3} unit of core polymerase (0.3µl of fraction C2) and 25×10^{-3} unit of specificity factor (0.1µl of fraction F8) for promoter characterization experiments. RNA products were analyzed by electrophoresis through 6% or 8% polyacrylamide, 7 M urea gels as described (4). Specific transcription was quantitated by cutting the relevant run-off transcripts from gel and liquid scintillation counting using "instagel" (Packard).

To correctly calculate the transcription efficiency of mutant promoters in combination clones, the ratio of label incorporated in mutant run-off and in the internal wild type control run-off was determined and divided by the ratio of test wild type run-off and control wild type run-off in the analogous wild type/wild type combination clone (see formula in table I and e.g. fig.4). Thus the efficiency of the mutant test promoter is compared to that of the wild type test promoter situated on an analogous DNA fragment, and corrected for possible variations between parallel reactions. Each combination clone was transcribed three times in independent reactions under identical conditions.

RESULTS

Introduction of point mutations in the large rRNA promoter

Point mutations were introduced in the M13 clone Mc21A-48/+46 which contains the large rRNA promoter region spanning from nucleotide -48 to +46 relative to the site of transcription initiation (+1, see figure 1). Oligonucleotide mixes containing each two or three different substitutions at a single nucleotide position were used for in vitro site-directed mutagenesis of single

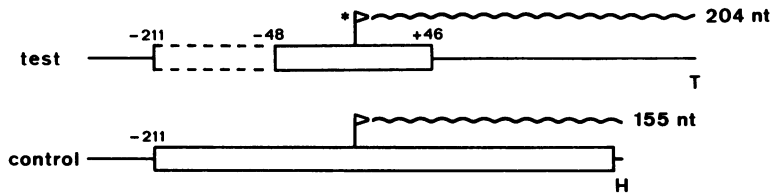

Figure 2: Structure and transcription of promoter containing DNA fragments resulting from digestion of pB21H-combination clones with TaqI and HindIII. The boxed regions indicate mtDNA sequences, the line vector sequences (pBR325, M13, linker). The stippled box region in the test fragment indicates the additional mtDNA sequence present in pB21H/-211/+46 clones relative to pB21H/-48/+46 clones. Mutated promoter sequences (*) if present are always on the test fragment. Run-off length from test promoters is 204 nt, from the control promoter 155 nt. The flag indicates the promoter, the wavy line RNA. H = HindIII, T = TaqI. The figure is not drawn to scale.

stranded DNA using a double-primer procedure as described (17,18). The substitutions ranged from position -2 to position +4. The mutants that we obtained from this and previous procedures (7) are presented in figure 1. To quantitate the effect of these mutations in an optimal manner all mutated promoter regions and analogous wild type promoter regions were cloned into the vector pB21H which already contains a wild type large rRNA promoter. The resulting clones were prepared for run-off transcription by digesting them with HindIII and TaqI, which results in separate DNA fragments with either the wild type promoter (control) or the mutated promoter (test). Each fragment supports the synthesis of a different run-off RNA measuring 155 nucleotides for the control promoter and 204 nucleotides for the test promoter (see figure 2). This approach ensures that control and test promoter are present in equimolar amounts in the transcription mixture and that there is no interference between transcription from the respective promoters. It also minimizes the possibility that small variations in the purification of the DNA templates interfere with quantitation of the mutant promoter efficiency.

Characterization of reaction conditions

We have characterized a number of variables of the transcription reaction in order to analyze the mutants under defined conditions.

Enzyme preparation. For analysis of the promoter mutants we used separate core polymerase and specificity factor containing fractions (14 and Schinkel et al., submitted) which were mixed for *in vitro* transcription reactions. First we analyzed the effect of varying the ratio of specificity factor to core polymerase. A fixed amount of core polymerase was used to transcribe the

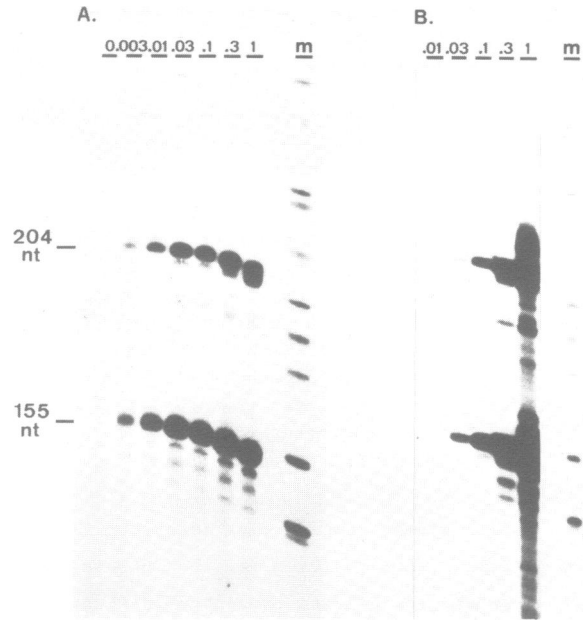

Figure 3: Dependence of specific transcription on ratio and concentration of specificity factor and core RNA polymerase.

A) Effect of increasing amounts of specificity factor. TaqI- and HindIII-digested pB21H/wt -48/+46 was transcribed with a constant amount of core RNA polymerase (7.10^{-3} unit, 0.3 µl of fraction C2) and with increasing amounts of specificity factor. 0, 0.003, 0.01, 0.03, 0.1, 0.3 and 1.0 µl of specificity factor fraction F8 (0.25 unit/µl) were added as indicated. M: DNA size marker (pBR322 digested with MspI). Size of test (204 nt) and control (155 nt) run-offs is indicated.

B) Effect of increasing amounts of core RNA polymerase. TaqI- and HindIII-digested pB21H/wt-48/+46 was transcribed with a constant amount of specificity factor (0.025 unit, 0.1 µl of fraction F8) and with increasing amounts of core RNA polymerase, 0.01, 0.03, 0.1, 0.3 and 1.0 µl of core polymerase fraction C2 (22.10^{-3} unit/µl) were added to the transcription mixture as indicated over the lanes. M and other symbols are as under A.

template pB21H/wt -48/+46 with increasing amounts of specificity factor and products of the reactions were analyzed by gel electrophoresis. Addition of up to 0.03 µl of factor fraction results in a clear increase of specific transcription from both wild type promoters and a concomitant decrease of non-specific transcription (fig.3A). When using a nearly saturating amount of factor fraction (0.1 µl), the amount of core polymerase is one of the limiting factors for specific transcription, as is demonstrated in figure 3B: increasing the amount of core polymerase leads to a continuous increase in the amount of run-off product. We observe that the relative intensity of the 204

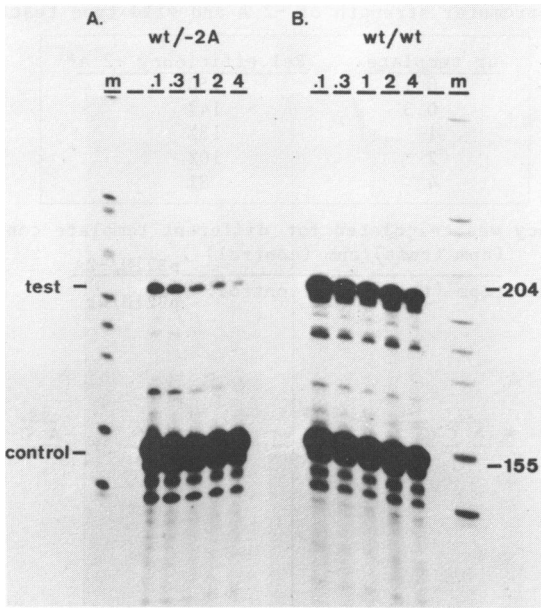

Figure 4: Analysis of competition between test and control promoters derived from one combination clone. TaqI- and HindIII-digested pB21H/-2A (panel A) or pB21H/wt-48/+46 (panel B) were transcribed with 0.1 µl of specificity factor fraction F8, and 0.3 µl of core polymerase fraction C2. The template concentration was varied from 0.1 µg to 4 µg per reaction as indicated over the lanes. Positions of test and control run-offs are indicated. M: DNA size marker (pBR322 digested with MspI).

nt run-off product is lower than that of the 155 nt product, although both are initiated from identical promoters (figs.3A and B). We do not exactly know the cause of this effect, but we have corrected for it in our promoter efficiency determinations (see below) by only comparing mutant promoters to a wildtype promoter situated on an analogous DNA fragment.

Concentration of template. Since the amount of (core) RNA polymerase is limiting in the transcription reaction under the conditions chosen an analysis of the wild type and mutant promoter simultaneously present in one reaction could be influenced by mutual competition for RNA polymerase components. To assess this effect we compared transcription at different promoter concentrations, from two different templates, pB21H/-2A containing a wild type control promoter and a weak -2A mutant test promoter (see below) and pB21H/wt -48/+46 containing two wild type promoters. The template concentration for each of the clones was raised from 0.1 to 4 µg DNA per reaction. The results depicted in figure 4 A and B show that even the lowest template concentration

TABLE I Relative promoter strength of -2 A and wild type test promoters.

ug template	Rel.efficiency -2 A*
0.1	15%
0.3	14%
1	13%
2	10%
4	8%

*Relative efficiency was calculated for different template concentrations using the formula:

$$\frac{(\text{cpm (test)/cpm (control)})_{\text{pB21H/-2A}}}{(\text{cpm (test)/cpm (control)})_{\text{pB21H/wt}}}$$

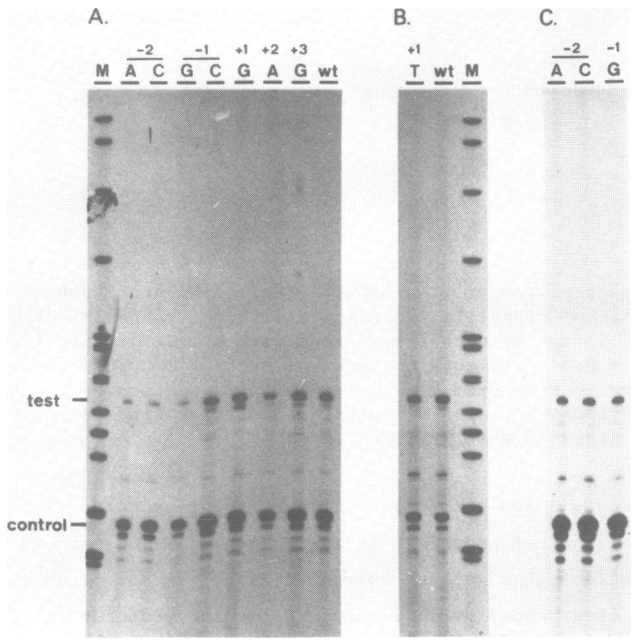

Figure 5: In vitro transcription from mutated and wild type promoters in pB21H/-48/+46 combination clones. TaqI- and HindIII-digested pB21H/mutant or pB21H/wild type clones were transcribed with 0.1 µl of specificity fraction F8 and 0.3 µl of core polymerase fraction C2.

A) -2 A, -2 C, -1 G, -1 C, +1 G, +2 A and +3 G mutant/wild type clones, and a wild type/wild type clone were transcribed using 10 µM α^{32}P-UTP (2 µCi per reaction) and 125 µM of other NTPs. The nature of the test promoter is indicated over each lane. M: DNA size marker (pBR322 digested with MspI).

B) +1 T mutant/wild type and a wild type/wild type clone were transcribed using 10 µM α^{32}P-GTP (2 µCi per reaction) and 125 µM of other NTPs. Indication of lanes as under A.

C) Prolonged exposure of lanes -2 A, -2 C and -1 G in panel A.

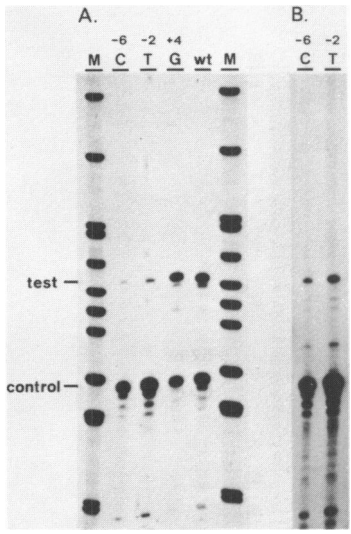

Figure 6: In vitro transcription from mutant and wild type promoters in pB21H/(-211/+46) clones. TaqI- and HindIII-digested pB21H/mutant and pB21H/ wild type clones were transcribed as in figure 5.
A) -6 C, -2 T and +4 G mutant/wild type clones and a wild type/wild type clone were transcribed using 10 µM α^{32}P-UTP (2 uCi per reaction) and 125 µM of other NTPs. The nature of the test promoter is indicated over each lane. M: see figure 5.
B) Prolonged exposure of lanes -6 C and -2 T in panel A.

is not limiting under these conditions, since there is no increase in run-off product upon increasing the amount of template. Furthermore, the weak run-off transcript from the -2A mutant promoter diminishes compared to the internal control wild type promoter. However, the wild type test run-off from the wild type/wild type clone shows the same effect, albeit to a lesser extent (fig.4B). A quantitative comparison between mutant and wild type test run-offs (see Table I) demonstrates that there is only a low level of direct competition between the test and control promoter under the conditions chosen for the promoter-mutant analysis (1 ug template per reaction). It thus becomes unlikely that a weak mutant promoter would be completely out-competed by the strong wild type promoter present as internal control.

Transcription efficiency of mutant promoters

All mutant promoters listed in figure 1 were tested for their ability to support specific transcription in vitro with digests of the pB21H combination clones at 1 ug per reaction, with 0.1 µl of specificity factor fraction F8 and 0.3 µl of core polymerase fraction C2. As demonstrated above, core polymerase is limiting under these conditions, and specificity factor nearly saturating. Figures 5A and B show that the mutant promoters -2 A, -2 C, -1 G, -1 C, +1 G, +2 A, +3 G and +1 T all still support specific transcription, albeit to a varying extent relative to the wild type reference clone (wt). The prolonged exposure of the rather weak -2A, -2C and -1G lanes in figure 5C more clearly reveals the specific transcripts from these mutated promoters.

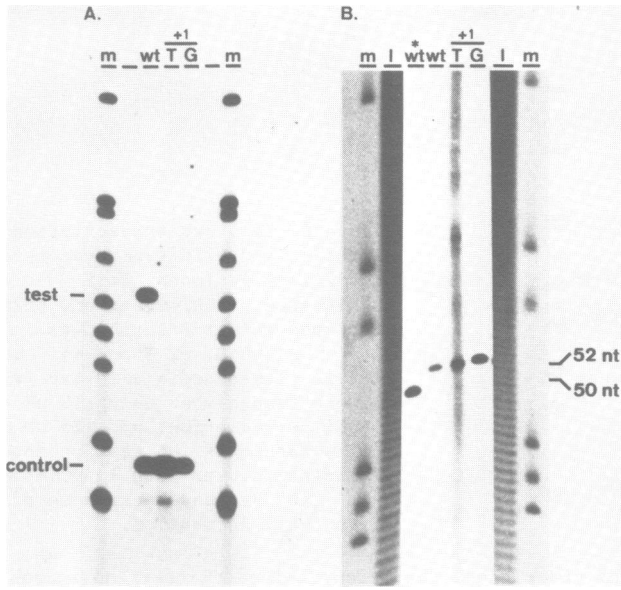

Figure 7: Analysis of transcription from +1 T and +1 G mutant promoters

A) HindIII-TaqI-digested pB21H/wt, pB21H/+1 T and pB21H/+1 G combination clones were transcribed as in figure 5, except that 20 µM of γ^{32}P-ATP (25 µCi per reaction) and 125 µM of other NTPs were used. The nature of the test promoter in each reaction is indicated over the lanes. Positions of test (204 nt) and control (155 nt) run-offs are indicated. M: see figure 5.

B) +1 T, +1 G and wild type (wt) promoter regions were excised from the original M13 clones and isolated by elution from agarose gel. Purified fragments were transcribed using either 10 µM α^{32}P-UTP and 125 µM of other NTPs (wt and +1 G) or 10 µM of α^{32}P-GTP and 125 µM of other NTPs (+1 T) and transcripts were analyzed on an 8% denaturing polyacrylamide gel. Initiation on the +1 nucleotide yields a 52 nt run-off product. A wild type fragment yielding a 50 nt run-off RNA product (wt*) and an alkaline degradation ladder of poly r(AT) (1) were electrophoresed in parallel to estimate the resolution of this experiment. M: DNA size marker (M13mp10 digested with DdeI). Length of run-off products is indicated.

The results of transcription of the combination clones containing mutant promoters present on longer (-211/+46) mtDNA fragments (see also fig.2) are presented in figure 6A and B. The -6 C and -2 T mutants are weakly, but still specifically transcribed, as can best be judged from the long exposure in panel B. Close comparison of the lanes marked +4 G and wt in panel A shows that the +4 G promoter mutant is somewhat more active than its analogous wild type promoter.

The efficient transcription from the +1 T and +1 G mutants is an interesting finding but before one can conclude that the identity of the initiating nucleotide is changed the possibility should be ruled out that the position at

TABLE II: Relative efficiency of mutant promoters

												Mean, standard deviation
								+1				
wt	A	T	A	T	A	A	G	T	A	G	T	A	100*) %
-6C	-	-	C	-	-	-	-	-	-	-	-	-	9 ± 2 %
-2T	-	-	-	-	-	-	T	-	-	-	-	-	10 ± 2 %
-2A	-	-	-	-	-	-	A	-	-	-	-	-	19 ± 3 %
-2C	-	-	-	-	-	-	C	-	-	-	-	-	20 ± 4 %
-1G	-	-	-	-	-	-	-	G	-	-	-	-	31 ± 2 %
-1C	-	-	-	-	-	-	-	C	-	-	-	-	88 ± 4 %
+1G	-	-	-	-	-	-	-	-	G	-	-	-	110 ± 9 %
+1T	-	-	-	-	-	-	-	-	T	-	-	-	57 ± 5 %
+2A	-	-	-	-	-	-	-	-	-	A	-	-	100 ± 12 %
+3G	-	-	-	-	-	-	-	-	-	-	G	-	92 ± 16 %
+4G	-	-	-	-	-	-	-	-	-	-	-	G	164 ± 5 %

*) Standard deviation for wt reference transcript was in the range of 2 to 6%.

which initiation takes place has shifted to one of the neighbouring A positions (-3 A or +4 A). To test this the +1 T and +1 G mutant combination clones were transcribed along with a wt/wt combination clone in a reaction mixture containing γ-^{32}P-ATP. Under these conditions only RNAs initiated with an A nucleotide will be labeled. Figure 7A shows that only transcripts from the wild type promoters are labeled, indicating that RNAs from +1 T and +1 G promoters do not initiate with ATP. Furthermore, the +1 T and +1 G mutant promoters were transcribed on isolated fragments supporting synthesis of very short (52 nt) run-off transcripts (Fig.7B). Comparison with the length of two wild type promoter transcripts (one of 52 and one of 50 nucleotides) and with an alkaline RNA degradation ladder indicates that the point of transcription initiation did not shift even one nucleotide. Thus, transcription from the +1 T and +1 G promoter mutants starts correctly at the +1 position.

The relative efficiency of each mutant promoter compared to the wild type promoter present on an analogous DNA fragment was determined from three independent transcription reactions as described in Materials and Methods. The relative efficiencies and standard deviations obtained from this analysis are presented in Table II. The data indicate that the -6 C and -2 T mutant promoters are the weakest, retaining approximately 10% of wild type promoter activity. The other two substitutions at position -2 result in about 20% of wild type activity. At position -1, the T to G transversion has a stronger effect (with about 30% of residual activity) than the T to C transition leaving about 90% of wild type activity. To our surprise, replacement of the initiating A by G does not affect transcription efficiency, whereas even a transversion to T causes only a moderate decrease. The two mutations analyzed at positions +2 and +3 do not significantly influence promoter efficiency but,

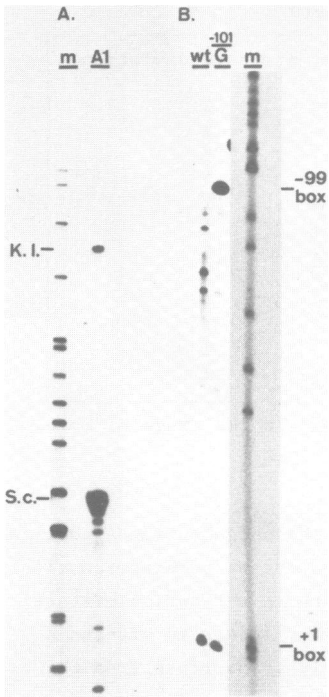

Figure 8:
A. Relative transcription of K.lactis and S.cerevisiae large rRNA promoters. A1:pB21H/20A1 was digested with HindIII and RsaI and transcribed as in figure 5. Specific run-off products from each promoter (K.l. = 348 nt, S.c. = 155 nt) are indicated. M: DNA size marker, pBR322 digested with HindIII.
B. Transcription from the -2 T or -2 G form of the homology box located 99 nucleotides upstream of the large rRNA promoter. SspI- and BamHI-digested pU21H (lane wt) and pU21 T/G^{-101} (lane -101 G) were transcribed as indicated in the legend to figure 5A. Specific run-off products from the +1 box (29 nt) or -99 box (128 nt) are indicated. M: see under A.

interestingly, an A to G transition at position +4 leads to a clear-cut rise in promoter efficiency. We have also tested the large rRNA promoter of Kluyveromyces lactis which contains the sequence ATATAAGT AATT. Figure 8A shows that the K.lactis promoter is only weakly transcribed in comparison to S.cerevisiae large rRNA promoter present as an internal control. We consider it likely that the T at position +4 causes this effect, also because a +4 T is not found in strong S.cerevisiae promoters (3,10).

Comparison of our data with those of others (8,9; see also discussion) suggests that sequences outside the region stretching from nucleotide -8 to +4 may also affect promoter efficiency. This is supported by the following observation: 99 nucleotides upstream of the large rRNA promoter a sequence ATATAATT AATA is found. With the exception of the presence of an A at position +2, which does not disturb the efficiency of the large rRNA promoter (Table II), this element is identical to the -2 T mutant which shows 10% of wild type promoter activity. However, at the position where the transcript from the -2 T box at position -99 in the unmutated clone would be expected, no specific signal can be detected (fig.8B, lane wt). When the -2 T in the -99 region is substituted by a G (7), a strong promoter is formed (fig.8B, lane -101G). It

thus appears that surrounding sequences can modulate the efficiency of promoter-like sequence elements.

DISCUSSION

We have analyzed the effect on transcription efficiency of point mutations introduced in the promoter region of the large rRNA gene of Saccharomyces cerevisiae mitochondrial DNA. To this purpose mutated promoter-fragments containing at least 45 nucleotides of mtDNA both upstream and downstream from the initiating nucleotide were transcribed in vitro with extensively and separately purified mtRNA polymerase components. Considering the very strong conservation of the originally detected consensus sequence of efficient natural mitochondrial promoters ($\frac{A}{T}$TATAAGT ApuTA) (2,3) this analysis has yielded some interesting results: In the context of the large rRNA promoter region none of the single point mutations analyzed by us completely abolishes promoter activity (see Table II).

Along the lines of expectation is that substitutions -6 C, -2 T, -2 A and -2 C which form part of the strongly conserved nonanucleotide box, have a strong negative effect on specific transcription. Especially the G nucleotide at position -2 distinguishes the core promoter element from the surrounding AT-rich intergenic sequences. However, all these mutant promoters still support a substantial level of transcription compared to the wild type activity (10 to 20%). This is partly in contrast to the data of Biswas and Getz (9) who analyzed transcription from -2 mutants introduced in a short synthetic promoter element which is homologous to nucleotides -13 to +4 of the small rRNA promoter and cloned in a bacterial vector. In their analysis all substitutions at position -2 completely abolished transcription activity. This apparent discrepancy might be attributable to differences in the enzyme preparations used, since entirely different purification procedures were applied, and neither preparation represents homogeneous mtRNA polymerase (Schinkel et al., submitted). Both enzyme concentration and ratio of specificity factor to core RNA polymerase could differ, and possibly additional, as yet unidentified proteins might differentially affect transcription from mutated promoters. However, we consider it more likely that sequences outside of the -8 to +4 region of the mitochondrial promoter can affect the ability of a mutilated consensus sequence to support specific transcription. We demonstrated such an effect for our transcription reaction conditions by comparing transcription form the inactive -2 T motif 99 nucleotides upstream of the large rRNA promoter with transcription from the weakly active -2 T mutant of the large rRNA promoter itself. We do not yet know what property of the surrounding

sequences is responsible for this effect, but it is probably not general AT-content, since this is comparable for the -99 motif and the +1 promoter. Some in vivo data also suggest a stimulating or inhibitory role of surrounding DNA sequences. For instance, in the petite mutant b7 containing the ori 2 (rep 3) replication origin, a nearly perfect consensus sequence (ATATAAGT AATT) some 20 nucleotides upstream of an active transcription initiation site does not give rise to detectable transcripts, whereas the poor consensus sequence AAATAAGT TAAA present in the same petite DNA does show some transcription initiation (19).

The behavior of the +1 G and +1 T mutants is interesting, because the +1 G promoter appears as active as the wild type promoter, whereas the +1 T mutation inhibits transcription only to a limited degree. mtRNA polymerase in both cases still uses the +1 position as initiating nucleotide, and it does not shift to a neighboring adenine (or other) position, as is for instance seen in the case of nuclear RNA polymerase III (20). Apparently in contrast to the situation for E.coli RNA polymerase there is no strong preference for purine as initiating nucleotide, and the configuration of the large rRNA promoter forces the mtRNA polymerase to the +1 nucleotide position for initiation. In this respect it is puzzling that a fairly good consensus sequence present in the yeast nuclear gal10 promoter region (C ATATAAGT AAGA, nucleotides not found in mitochondrial promoters are underlined) supports transcription from the -1 T position (11). Possibly in this case too surrounding sequences can affect specific transcription.

Biswas and Getz (8) have systematically analyzed the effect of substitutions at positions +2 and +3 of the synthetic small rRNA promoter element. The mutations obtained by us in this area do not allow a full-scale comparison, but whereas the small effect of the G to A mutation at position +2 in the large rRNA promoter mirrors the findings in the synthetic promoter, the activity of our +3 G mutant is relatively higher (92% of wild type level) than that of the +2 G/+3 G mutant synthetic promoter (52%). Possible explanations for this discrepancy could again invoke differences in the enzyme preparations used, and differential effects of surrounding sequences as discussed above.

The A at position +4 that was mutagenized forms the final nucleotide of the puTA motif that is conserved in strong promoters (3). Two separate findings further indicate that the +4 nucleotide has a significant role in modulation of transcription efficiency: The clear increase in activity of the S.cerevisiae large rRNA promoter by an A to G transition at position +4, and the relatively weak transcription of the K.lactis large rRNA promoter

containing a T at position +4. Although the +4 G mutant is the only clear promoter-up mutation that we have detected so far, there are no known natural mitochondrial promoters possessing a G at this position (3). This could be coincidental, but maybe in vivo additional requirements prevent the occurrence of a G at this position.

In general, the analysis of the mutations described here does not suggest that promoter efficiency is affected by the energy required for DNA strand dissociation. For instance, replacement of the +1 A:T basepair by G:C does not decrease the efficiency of transcription initiation. Also, at position -1 replacement of T:A by C:G has little effect, whereas G:C causes inhibition, suggesting that some other variable is more important in these cases. The process of transcription initiation by RNA polymerase can be divided into a number of subsequent steps, including promoter sequence recognition and binding, formation of an open complex, initiation of RNA synthesis and transition into an elongating complex (see e.g. ref. 21). A much more detailed dissection of the initiation process will be required to determine which step(s) are affected by each of the mutations that we have generated.

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Abbreviations: mt, mitochondrial; nt, nucleotide; NTP, ribonucleoside triphosphate; dsDNA, double stranded DNA; ssDNA, single stranded DNA; rRNA, ribosomal RNA; pu, purine.

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