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Characterization of the promoter of the large ribosomal RNA gene in yeast mitochondria and separation of mitochondrial RNA polymerase into two different functional components

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We have characterized a DNA sequence that functions in recognition of the promoter of the mitochondrial large rRNA gene by the yeast mtRNA polymerase. Promoter-containing DNA fragments were mutagenized and used as templates to study initiation of transcription *in vitro* with a partially purified mtRNA polymerase preparation. Deletion mutants, in which increasing stretches of DNA were removed from regions flanking the promoter, define a short area essential for correct initiation of transcription. It virtually coincides with a highly conserved stretch of nine nucleotides that is found immediately upstream of all transcriptional start sites described thus far. Two different point mutations within this nonanucleotide sequence drastically reduce promoter function. Conversely a single point mutation that results in the formation of a nonanucleotide sequence 99 nucleotides upstream of the large rRNA gene leads to a new, efficient transcription initiation site. MtRNA polymerase can be resolved into two different components by chromatography on Blue Sepharose: one retaining the capacity to synthesize RNA, the other conferring the correct specificity of initiation to the catalytic component.

Key words: mitochondria/promoter/RNA polymerase/transcription/yeast

Introduction

The mitochondrial genome of *Saccharomyces cerevisiae* consists of ~80 kb and encodes a number of genes whose products are components of inner membrane respiratory chain complexes, function in protein synthesis or are involved in various forms of RNA processing (reviewed by Dujon, 1981). The existence of multiple promoters was first inferred from the observation that several mtRNA species, including rRNAs, contain a 5'-triphosphate end as trademark of transcriptional initiation (Levens *et al.*, 1981b). DNA sequence comparison between the regions in which the 5' ends of the rRNA primary transcripts map revealed the presence of a strongly conserved sequence of nine nucleotides: ATATAAGTA (Osinga and Tabak, 1982; Osinga *et al.*, 1982). Alignment of the 5'-terminal sequence of the rRNA primary transcripts (Levens *et al.*, 1981b; Christianson *et al.*, 1982) and the DNA sequence showed that rRNA synthesis is initiated at the last nucleotide of this nonanucleotide box (Osinga *et al.*, 1982). The importance of the nonanucleotide box is emphasized further by the fact that almost all transcriptional starts identified thus far on *S. cerevisiae* mtDNA occur at the 3'-ultimate position of this sequence motif (Christianson and Rabinowitz, 1983) and by the fact that the same motif is found at the initiation sites of rRNA synthesis in the distantly related yeast *Kluyveromyces lactis* (Osinga *et al.*, 1982). Certain genes lack the nonanucleotide box but these turn out to be co-transcribed with genes located upstream that do possess the box sequence (Tabak *et al.*, 1983; Miller *et al.*, 1983; Christianson *et al.*, 1983; Osinga *et al.*, 1984; Zassenhaus *et al.*, 1984). We therefore proposed that the nonanucleotide sequence is an important part of the mitochondrial promoter.

The availability of a correctly initiating *in vitro* transcription system (Edwards *et al.*, 1982) allowed us to test critically this proposal. To this end we have introduced deletions and mutations in and around the nonanucleotide box and studied their effect on initiation of RNA synthesis. In this report we show that essential sequences necessary for *in vitro* promoter recognition indeed largely coincide with the conserved nonanucleotide sequence motif. Some of these findings were recently reported in preliminary form (Tabak *et al.*, 1985). Our results are in agreement with the characterization of the promoter of the small rRNA gene (Biswas *et al.*, 1985).

We have further characterized the mitochondrial RNA polymerase by chromatography on Blue Sepharose and describe the separation of the enzyme into two functional components.

Results

Promoter demarcation by deletion mapping

We have subcloned a mtDNA fragment containing the transcription initiation site for synthesis of large rRNA and used it for the construction of deletion mutants. This fragment contains 48 bp upstream and 46 bp downstream of the initiation site (Figure 1). It supports correct initiation of transcription in an *in vitro* system containing partially purified mtRNA polymerase (fractions from the second phosphocellulose column of procedure I, see Materials and methods) under conditions described by Edwards *et al.* (1982). Prior to transcription the DNA was truncated with a restriction enzyme to yield a fragment ending at a fixed distance from the site of RNA initiation. Correct initiation then leads to synthesis of RNA with predictable length. This is illustrated in Figure 3, lane b in which the 249-nucleotide RNA is the run-off product from the template Mc21A−48/+46 shown in Figure 1 truncated by *Pvu*II. Sequence analysis of the 5' end of this RNA indicates that initiation takes place at the last nucleotide of the nonanucleotide box (not shown). For construction of 5' deletion mutants the DNA clone Mc21B−48/+46 shown in Figure 1 was cut with *Eco*RI and the resulting ends were shortened by treatment with exonuclease *Bal*31. After ligation of *Bam*HI linkers to the ends, the collection of DNA fragments was re-cloned in the M13 vector mp18, for further details see Materials and methods. A similar set of 3' deletion mutants was prepared by

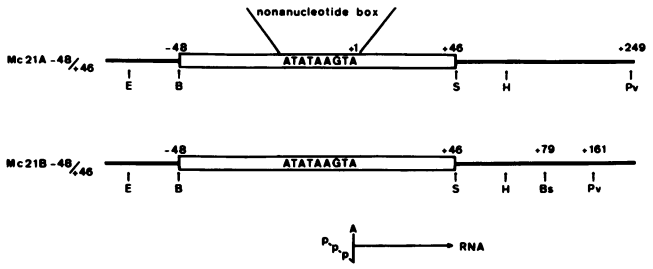


Fig. 1. Schematic representation of two M13 clones containing the yeast mtDNA fragment which carries the promoter for synthesis of large ribosomal RNA. Mc21A−48/+46 is a clone in M13mp19, Mc21B−48/+46 in M13mp18. The conserved nonanucleotide box is indicated. The boxed region represents mtDNA, the bold line vector DNA. Restriction sites used for run-off transcription (Figures 3 and 4) and their position relative to the first transcribed nucleotide (+1) are indicated. B = *Bam*HI, Bs = *Bsp*RI, E = *Eco*RI, H = *Hin*dIII, Pv = *Pvu*II and S = *Sal*I.

3′ DELETION BANK

	NONANUCLEOTIDE BOX +1	PROMOTER ACTIVITY
Mc21A -48/+17	TTATTATATATATAAGTAGTAAAAAGTAGAATAAGGTCGACC	+
-48/+11	TTATTATATATATAAGTAGTAAAAAGTAGGTCGACC	+
-48/+8	TTATTATATATATAAGTAGTAAAAAGGTCGACC	+
-48/+5	TTATTATATATATAAGTAGTAAGGTCGACC	+
-48/+4	TTATTATATATATAAGTAGTAGGTCGACC	+
-48/+3	TTATTATATATATAAGTAGTGGTCGACC	+
-48/+2	TTATTATATATATAAGTAGGTCGACC	+
-48/-2	TTATTATATATATAAGGGTCGACC	-
-48/-2′	TTATTATATATATAAGGTCGACC	-
-48/-4	TTATTATATATATAGGTCGACC	-
-48/-5	TTATTATATATATGGTCGACC	-

5′ DELETION BANK

Mc21B -17/+46	GGATCCGGGTTATTATATATATAAGTAGTAAAAAGTAGAATAA	+
-14/+46	GGATCCGGGTTATATATATAAGTAGTAAAAAGTAGAATAA	+
-13/+46	GGATCCGGGTATATATATAAGTAGTAAAAAGTAGAATAA	+
-11/+46	GGATCCGGGTATATATAAGTAGTAAAAAGTAGAATAA	+
- 9/+46	GGATCCGGGTATATAAGTAGTAAAAAGTAGAATAA	+
- 7/+46	GGATCCGGGTATAAGTAGTAAAAAGTAGAATAA	+
- 6/+46	GGATCCGGGATAAGTAGTAAAAAGTAGAATAA	-
- 4/+46	GGATCCGGGAAGTAGTAAAAAGTAGAATAA	-
- 2/+46	GGATCCGGGGTAGTAAAAAGTAGAATAA	-
- 2′/+46	GGATCCGGGGTAGTAAAAAGTAGAATAA	-
+ 1/+46	GGATCCGGGAGTAAAAAGTAGAATAA	-

Fig. 2. Compilation of the DNA sequence of the various *Bal*31 deletion mutants combined with their effects on transcription-initiation. *Bal*31 deletion mutants were sequenced as described in Materials and methods. Mutants still supporting the synthesis of the predicted run-off product are scored + for promoter activity. A prime indicates that two different deletion clones contain the same mtDNA sequence due to replacement of a mtDNA G residue by a linker G residue and only differ in the flanking sequence.

opening Mc21B−48/+46 with *Hin*dIII at a position downstream of the promoter and using *Sal*I linkers to re-clone the remaining mtDNA fragments.

The exact length of the deletions was determined by DNA sequence analysis. Linkers with high GC content were chosen to ensure a substantial difference from the original AT-rich nucleotide sequence at the borders of the deletions, in an attempt to minimize the possibility of a (partial) take-over of promoter function by the replacing DNA. The set of deletions obtained is displayed in Figure 2.

Analysis of the resulting deletion mutants was carried out as follows. DNAs of the 3′ deletion bank and wild-type DNA were

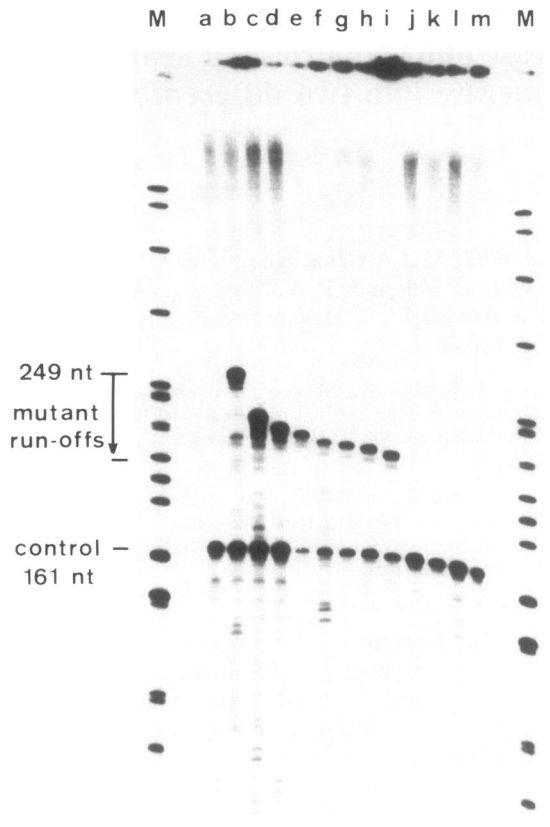


Fig. 3. *In vitro* transcription of 3′-deletion mutants. *Pvu*II truncated Mc21A−48/+46 (see also Figure 1) gives rise to a run-off RNA of 249 nucleotides (**lane b**). **Lanes c−m** represent transcription results from progressively deleted templates depicted in Figure 2. Mc21B−48/+46 (opposite orientation) was cut with *Pvu*II to yield a transcript of 161 nucleotides, see **lane a**. This DNA was present in each reaction to serve as internal control for transcription-initiation. Templates used in **lanes c−m** were respectively deleted up to positions: **c**: +17, **d**: +11/12, **e**: +8/9, **f**: +5, **g**: +4, **h**: +3, **i**: +1/2, **j**: −2, **k**: −3/2, **l**: −4, **m**: −5. M: DNA size marker (pBR322 digested with *Msp*I and 5′-end labelled).

each cleaved by a restriction enzyme to yield distinguishable run-off RNAs. The wild-type DNA was mixed into each transcription reaction to serve as internal control. After transcription and analysis of the RNA products by polyacrylamide gel electrophoresis, we observe in all lanes the 161-nucleotide wild-type run-off product (Figure 3). RNA synthesis from the 3′-deleted templates stops when the deletion goes from position +2 to position −2. Note that removal of gene internal base pairs results in shorter transcripts. A similar analysis was carried out on the mutant templates containing the 5′ deletions (Figure 4). In this case the internal control template promotes the synthesis of a 79-nucleotide RNA product. The mutant DNA templates support synthesis of a 161-nucleotide run-off RNA until the deletion eliminates the nucleotide at position −7. Deletions that include nucleotide −7 or extend beyond it result in a dramatic decrease of specific run-off transcript production. This implies that the first nucleotide of the nonanucleotide box can be replaced by a G without noticeable effect on correct initiation of transcription. Interpretation of the results obtained with the DNAs from the 3′ deletion bank is complicated by the fact that in some cases a G deleted from the mtDNA sequence is replaced by one derived from the *Sal*I linker. The results of the deletion demarcation experiments have been summarized in Figure 2. We conclude from this qualitative analysis that the region spanning

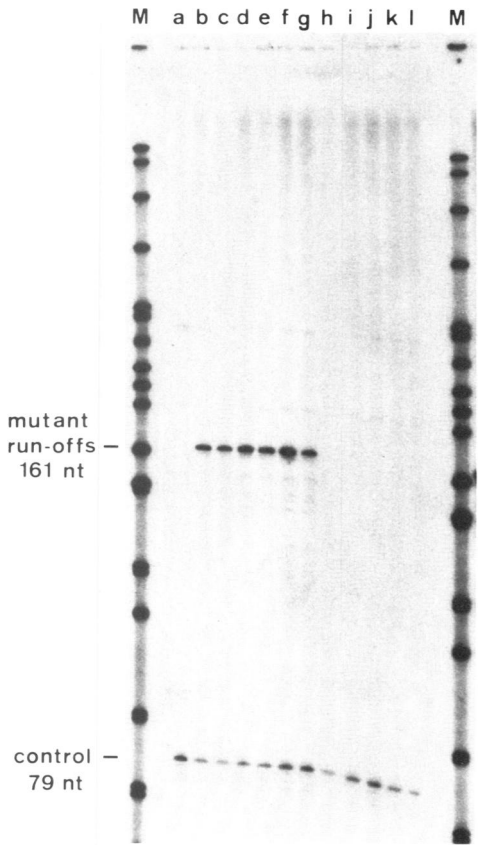
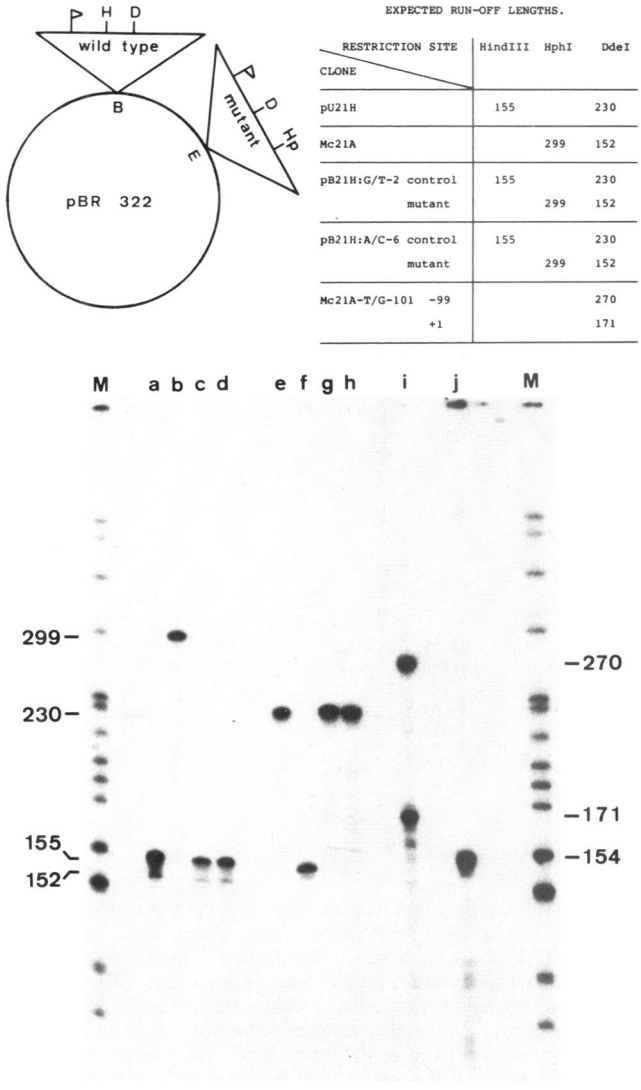


Fig. 4. *In vitro* transcription of 5′ deletion mutants. *Pvu*II truncated Mc21B 5′ deletion clones give rise to an expected run-off RNA of 161 nucleotides (**lane b**). **Lanes b–l** represent transcription results from progressively deleted templates (see Figure 2). Mc21B−48/+46 cut by *Bsp*RI served as internal control for transcription-initiation (expected run-off length 79 nucleotides), see **lane a**. Templates used in **lanes b–l** were respectively deleted up to positions: **b**: −17, **c**: −14, **d**: −13, **e**: −11, **f**: −9, **g**: −7, **h**: −6, **i**: −4, **j**: −2, **k**: −1/−2, **l**: +1.



CLONE \ RESTRICTION SITE	HindIII	HphI	DdeI
pU21H	155		230
Mc21A		299	152
pB21H:G/T-2 control	155		230
mutant		299	152
pB21H:A/C-6 control	155		230
mutant		299	152
Mc21A-T/G-101 -99			270
+1			171

Fig. 5. *In vitro* transcription of nonanucleotide containing mtDNA fragments with single basepair alterations (see Materials and methods). The diagram shows the schematic structure of pBR322 clones containing both 'wild type' and mutagenized 21S promoter regions. The nonanucleotide region is indicated by a flag. The table indicates expected run-off lengths for the different clones and double clones used. B = *Bam*HI, D = *Dde*I, E = *Eco*RI, H = *Hin*dIII and Hp = *Hph*I are restriction sites. **Lane a**: transcription of wild-type promoter containing pU21H cut by *Hin*dIII which gives rise to a run-off RNA of 155 nucleotides. **Lane b**: as for **lane a** but Mc21A was cut by *Hph*I which gives rise to a 299 nucleotide run-off RNA. **Lane c**: *in vitro* transcription of pB21H:21G/T^{-2} containing both the wild-type 21S rRNA promoter and the G to T mutated box at position −2. The clone was cut by *Hin*dIII and *Hph*I. Expected run-off RNA lengths for wild-type and mutant promoter are 155 and 299 nucleotides respectively. **Lane d**: as in **c**, but here the transcribed clone (pB21H:21A/C^{-6}) contains besides the wild-type promoter a mutated box having C instead of A at position −6. In **lanes e, f, g** and **h** the same set of DNAs described in **a, b, c**, and **d** respectively was analysed but this time after DNA truncation by *Dde*I. The run-off product from the wild-type control is 230 nucleotides. The products expected from the mutated nonanucleotide boxes are 152 nucleotides. Note: the alteration of the A at position −6 into the C led to formation of a *Dde*I cleavage site. In **lane h** the DNA template therefore only retains the 3′-part of the nonanucleotide box. **Lane i**: transcription of Mc21A-T/G^{-101} containing a second nonanucleotide box at position −99 upstream of the wild-type 21S rRNA promoter due to a T to G transversion at position −101. The *Dde*I truncated template gives rise to a run-off product of 171 nucleotides from the wild-type box and a 270 nucleotide RNA product initiated at the artificial box. **Lane j**: transcription of a *Kluyveromyces lactis* mtDNA clone (Ml15A) containing the transcription initiation site for small rRNA truncated by *Rsa*I. Expected run-off product: 154 nucleotides.

nucleotide −7 to −1/+2 is required for correct *in vitro* transcription initiation on the 21S rRNA gene promoter. These results are in good agreement with similar demarcation studies by Biswas *et al.* (1985), who localized the 5′ border of the 15S rRNA gene promoter between position −10 and −5, and the 3′ border between positions −1 and −3. The −7 to −1 region coincides to a large extent with the conserved nonanucleotide box previously found at the majority of transcription initiation sites.

Point mutations

Following another line of experimentation we have introduced single nucleotide substitutions in the nonanucleotide box by *in vitro* site-directed mutagenesis. In one experiment the G at position −2 was altered into a T, in another the A at position −6 into a C and the effect of these mutations on initiation of transcription was studied (Figure 5). Since we have noticed that transcription results can be influenced by the quality of the DNA preparation used, we have cloned the DNA containing these altered nonanucleotide boxes in a plasmid already containing the wild-type promoter (see diagram in Figure 5). In this way both promoters are present in equal amounts in the transcription mixture and factors that influence transcription should affect both to the same extent. Recombinant plasmids were cut with *Hin*dIII and *Hph*I in order to obtain run-off RNAs of different lengths and to have each promoter on a different fragment, thus preventing possible interference during transcription. In Figure

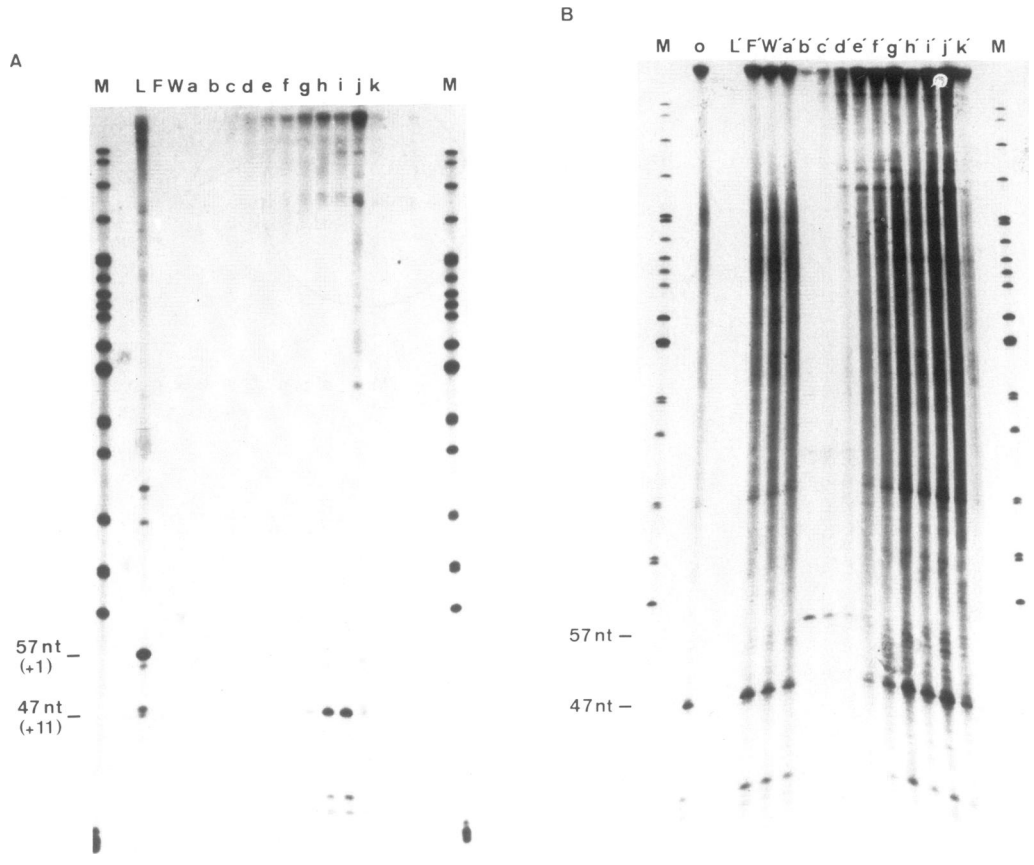


Fig. 6. Chromatography of a partially purified mtRNA polymerase preparation on Blue Sepharose: separation in two functionally different components. **A**: fractions were assayed for RNA polymerase activity by using a mtDNA clone (pU21H) containing the promoter for synthesis of large rRNA. In the absence of CTP, RNA synthesis stops at the first C behind the point of transcription-initiation. The enzyme preparation used for this experiment gives rise to a discrete 57 nucleotide RNA product initiated at position +1 within the nonanucleotide box (fraction L). Fractions 20–32 (**lanes f–j**) also display some specificity of initiation but the RNA is shorter (47 nucleotides) and is initiated at position +11 downstream of the nonanucleotide box. **B**: the same fractions as in **A** are assayed on the same template in the presence of an enzyme preparation displaying only +11 initiating activity but which is derived from another purification method (Procedure II). Fractions 8 – 14 (**lanes b′** to **d′**) contain a factor(s) which leads to reconstitution of +1 initiation specificity. Respective lanes represent transcription results from the following Blue Sepharose fractions: L: load, F: flow-through, W: wash fraction. **a**:4, **b**:8, **c**:12, **d**:14, **e**:18, **f**:20, **g**:22, **h**:26, **i**:30, **j**:32, **k**:36. M: mol. wt marker. O represents transcription by a +11 initiating fraction from procedure II. Lanes marked with a prime indicate that the reactions were supplemented with this fraction.

5 wild-type controls are shown to indicate the positions of run-off products obtained after transcription of *Hin*dIII-truncated DNA (155 nucleotides, lane a) or *Hph*I-truncated DNA (299 nucleotides, lane b). When *Hin*dIII and *Hph*I are used together for digestion of the recombinant DNA clones containing a mutant and wild-type promoter, *in vitro* transcription yields only the run-off product from the wild-type promoter (155 nucleotides). From the mutated promoters no run-off RNA can be detected (Figure 5, lanes c and d). The analysis was repeated using the enzyme *Dde*I (Figure 5, lanes e–h). In this case the wild-type promoter supports the synthesis of the longer run-off RNA (230 nucleotides, lane e) while the mutated promoter is expected to yield the shorter run-off product of 152 nucleotides. Again, no RNA product derived from the mutated promoters can be discerned (see for mutant A–C at position –6 the remark in the legend to Figure 5). Each of the two nucleotides of the nonanucleotide box that were altered in these experiments thus plays an essential role in promoter recognition by mtRNA polymerase.

The importance of the nonanucleotide box can also be demonstrated in another way. The region upstream of the 21S rRNA promoter region is completely silent with regard to initiation of transcription (see for example lane a of Figure 5). We have changed a T at position –101 into a G, thus generating the sequence TATA ATATAAGTA ATA containing a nonanucleotide box 99 nucleotides upstream of the normal 21S rRNA box. This artificial box has a 3′-flanking sequence which conforms to the extended conserved sequence motif originally noticed by Christianson and Rabinowitz (1983). The result of *in vitro* transcription of this construct is shown in Figure 5, lane i. Two run-off RNAs are found in roughly equal amounts. One has a length of 171 nucleotides and corresponds to the product derived from initiation at the naturally occurring nonanucleotide box. The other is a 270-nucleotide RNA derived from initiation at position –99. Characterization of the 270-nucleotide RNA by digestion with T1 ribonuclease shows that initiation takes place at the last nucleotide of the newly created nonanucleotide box (experiment not shown). This single nucleotide alteration therefore results in the formation of a promoter that is efficiently recognized by mtRNA polymerase.

We have previously reported that nonanucleotide boxes are strongly conserved and present in front of the rRNA genes in mtDNA of the distantly related yeast *K. lactis* (Osinga *et al.*, 1982). A DNA fragment containing the nonanucleotide sequence belonging to the small rRNA gene of this strain was used as template for *in vitro* transcription. It clearly promotes synthesis of the expected run-off RNA (Figure 5, lane j).

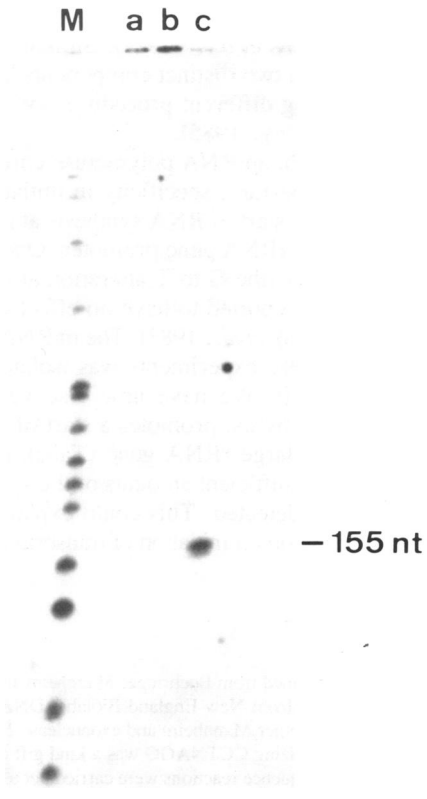


Fig. 7. Reconstitution of correct transcription initiation. Equal amounts of Blue Sepharose fractions 8 and 30 were mixed and used to transcribe *Hin*dIII truncated pU21H in a run-off assay. The correct 155 nucleotide RNA product is formed (**lane c**). The single fractions show little or no specific transcription-initiation (**lane a** fraction 8, **lane b** fraction 30). Note: +11 transcription is hardly detectable when using this template in run-off assays.

Separation of mtRNA polymerase into two different components

We have further characterized the partially purified mtRNA polymerase by chromatography on a Blue Sepharose column. When fractions eluted by a KCl gradient are assayed in the absence of CTP for RNA polymerase activity using a fragment containing the promoter for the large rRNA gene, fractions are found that support synthesis of a discrete short RNA fragment terminating at the position at which the first CTP must be incorporated (Figure 6A, fractions 22−32, lanes g−j). RNA sequence analysis, however, shows that synthesis of this RNA is the result of RNA initiation at position +11, 10 nucleotides downstream of the normal site of RNA initiation (not shown). The preparation that was loaded onto the column initiates RNA synthesis at the correct position as judged from the size of the run-off product (Figure 6A, lane L). When the column eluates were assayed in the presence of an aliquot of fractions that display +11 initiating activity, the results of Figure 6B are obtained. Correctly initiated RNA species are produced by fractions 8−14, fractions that have no activity without the extra addition (compare lanes b′, c′ and d′ of Figure 6B with lanes b, c and d of Figure 6A). Interestingly, these same fractions also suppress aspecific starts of RNA synthesis. For convenience we have used for this experiment an enzyme preparation which was much more active in +11 initiating activity than fractions 22−32 of the Blue Sepharose column, but which was derived from another purification procedure, procedure II (see Materials and methods and Discussion). When the most active fraction displaying +11 initiating activity of the Blue Sepharose column itself is combined with a column fraction containing the +1 conferring activity, the same phenomenon is observed: initiation of RNA synthesis at position +1 is restored (Figure 7).

We conclude that upon chromatography on Blue Sepharose, the mtRNA polymerase is separated into two components each eluting at a different salt concentration from the column: a component (A) which displays aberrant initiation of RNA synthesis and a component (B) which confers proper specificity in RNA initiation to component A.

Discussion

Initiation of transcription on mtDNA of *S. cerevisiae* takes place in most cases at the last nucleotide of a conserved stretch of nucleotides ($\frac{A}{T}$TATAAGTA): nonanucleotide box. Based on this observation we proposed that this sequence motif could be part of a yeast mitochondrial promoter. This is confirmed by the *in vitro* transcription studies on the various mutated forms of the large rRNA gene promoter that we report here. An essential part of the promoter as delineated with the aid of *Bal*31 deletion mutants approximately coincides with the nonanucleotide box, as was also shown by Biswas *et al.* (1985) for the small rRNA gene promoter. Single nucleotide alterations at two different positions within the nonanucleotide box drastically reduce initiation of transcription, while a single point mutation which creates a nonanucleotide box in an otherwise silent region of the mtDNA leads to formation of an active initiation site of RNA synthesis. The precise borders of the promoter cannot be established from deletion mapping only, since there is always a possibility that the DNA replacing the mtDNA sequence restores to some extent the function encoded in the deleted part. This is particularly so for the demarcation of the 3′ boundary of the promoter. The lack of −1 and +1 deletion mutants in the deletion bank and the presence of a G at position +2, which can be replaced by the linker DNA, do not allow us to draw conclusions as to the importance of base pairs at positions +1 and +2 (Figure 2).

The yeast mitochondrial promoter is remarkable when compared with others by its rather invariant nucleotide sequence. Nonanucleotide boxes are not only present in front of many mitochondrial genes but also in replication origins which promote RNA priming of DNA synthesis (Osinga *et al.*, 1982; Baldacci *et al.*, 1984) and they are found in the distantly related yeast strains *K. lactis* and *Torulopsis glabrata* at positions of RNA initiation (Osinga *et al.*, 1982; Clark-Walker *et al.*, 1985).

RNA extracted from mitochondria contains gene transcripts varying greatly in steady state concentration. It seems likely that at least part of this phenomenon is a consequence of a difference in initiation rate of these RNAs from their corresponding promoter. This would be at odds with the invariant composition of the mitochondrial promoters described thus far and could indicate that other nucleotides apart from the nonanucleotide box affect promoter function. This is also suggested by the finding that nonanucleotide sequences have been found that do not appear to give rise to detectable transcription initiation *in vivo* (Christianson and Rabinowitz, 1983; Colin *et al.*, 1985). Indications for the importance of nucleotides outside the nonanucleotide box came from the analysis of the promoters belonging to the gene coding for ATPase subunit 9. This gene is preceded by two nonanucleotide boxes, the first of which is much more active in initiation of transcription than the second, both *in vitro* and *in vivo* (Edwards *et al.*, 1983). In preliminary experiments we have also observed such differences when a recombinant DNA containing promoters from two different genes was used for *in*

vitro transcription. When the nucleotide sequences of all readily detectable promoters are compared there is also some conservation of nucleotide sequence downstream of the box: TATAAGTApuTA (Christianson and Rabinowitz, 1983). The second nonanucleotide box of the ATPase subunit gene (ATATAAGTATAT) deviates substantially from the consensus in this area. An analogous situation exists for the $tRNA^{f\text{-}met}$ gene, which is also preceded by two boxes, of which only the most active one conforms to the extended conservation (Miller *et al.*, 1983; Christianson and Rabinowitz, 1983). We therefore expect that nucleotide sequences modulating promoter function are most likely to be found immediately downstream of the nonanucleotide box. In this context it is worth noting that the efficient artificial promoter introduced in front of the 21S rRNA box also carries the puTA motif at its 3′ end. A more subtle and quantitative analysis will be needed to identify sequences determining promoter efficiency.

A few cases have been described which suggest that transcription initiation sites do not necessarily need to possess a nonanucleotide box. In a petite mutant transcription initiation has been demonstrated within the sequence motif AAATAAGTT AAA located in front of URF5 (Colin *et al.*, 1985). It differs from the nonanucleotide box at positions −7 and +1 and at position +3 with respect to the puTA consensus. The efficiency of this promoter is low compared with that of the ori2-r promoter (ATATAAGTA ATA) present in the same petite. By S1 nuclease protection analysis the 5′ end of the precursor $tRNA_2^{thr}$, accumulating in a petite mutant, has been mapped at a promoter-like sequence (GTATAAGTA TAC). It remains to be shown, however, whether the 5′ end originates from processing or from transcription initiation (Palleschi *et al.*, 1984). The promoter in front of the $tRNA^{glu}$ gene has been reported to contain a G at position −3 instead of an A (Christianson *et al.*, 1983). We have sequenced this region in *S. cerevisiae* strain D273-10B and found an A at position −3. As long as the necessity of an A at this position has not been demonstrated it may be considered to be a strain polymorphism.

In vitro transcription studies also suggest that deviations from the nonanucleotide box may be tolerated. Recently we have altered the A at position +1 into a G. Notwithstanding the almost absolute conservation of the A at this position, this mutated promoter can still support some synthesis of the predicted run-off RNA product. We are currently determining the RNA sequence at the 5′ end of this RNA product to see whether initiation indeed occurred at position +1 with GTP as the first nucleotide. This finding is in line with the observation of Biswas *et al.* (1985) that a *Bal*31 deletion mutant which introduced a G residue at position +1 of the 15S rRNA promoter could still support some initiation. Together with the permissible replacement of the A/T at position −8 by a G in our 5′ deletion mutant, these observations also suggest that *in vitro*, not every base pair within the nonanucleotide box contributes equally to promoter recognition.

Further purification of crude mtRNA polymerase preparations by chromatography on Blue Sepharose has led to separation into two components: one retaining the capacity to synthesize incorrectly initiated RNA chains, and one which provides specificity in the initiation of RNA synthesis when combined with the first component. This behaviour is rather similar to the separation of the *Escherichia coli* RNA polymerase into a sigma factor and core RNA polymerase upon chromatography on phosphocellulose. The separation of mtRNA polymerase into two components with different chromatographic and functional properties is not in agreement with an earlier characterization in which it was proposed that the enzyme consists of two identical subunits of 45 kd (Levens *et al.*, 1981a). Similar resolution of mtRNA polymerase into two distinct components has also been observed by others using different procedures (Winkley *et al.*, 1985; Levens and Howley, 1985).

It is remarkable that the mtRNA polymerase core component retains some, though aberrant, specificity in initiation of RNA chains as reflected in the start of RNA synthesis at position +11 downstream of the large rRNA gene promoter. One of the point mutations within the box (the G to T alteration at position −2) was studied before and reported to have no effect on the initiation of transcription (Tabak *et al.*, 1983). The mtRNA polymerase preparation used for these experiments was isolated according to Levens *et al.* (1981a). We have now discovered that this preparation, as purified by us, promotes a start of transcription at position +11 of the large rRNA gene (Tabak *et al.*, 1985) and probably contains insufficient amounts of the specificity conferring factor we have detected. This could explain the lack of effect of this point mutation on initiation of transcription described in this earlier report.

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, DNA polymerase I, Klenow fragment from Boehringer Mannheim and exonuclease *Bal*31 from New England Biolabs. *Aoc*I recognizing CCTNAGG was a kind gift of Dr de Waard (State University, Leiden). Sequence reactions were carried out using the dideoxy chain termination method (Sanger *et al.*, 1977).

Recombinant DNA techniques were carried out according to standard procedures (Maniatis *et al.*, 1982).

Recombinant plasmids

Mc21A−48/+46 and Mc21B−48/+46. The M13 clone Mc21A (Osinga *et al.*, 1983) was digested with restriction endonuclease *Aha*III after which *Sal*I linkers (GGTCGACC) were ligated to the isolated 700-bp *Aha*III fragment. After digestion with *Bam*HI and *Sal*I a 262-bp subfragment, containing 46 bp of the 21S rRNA gene and 209 bp of the 5′-flanking sequence, was isolated and cloned in *Bam*HI-*Sal*I-restricted M13mp18 (Mc21B−209/+46). In order to shorten the upstream region Mc21B−209/+46 DNA was opened at the *Bam*HI site and incubated with exonuclease *Bal*31. After ligation of *Bam*HI linkers (CCCGGATCCGGG), isolated *Bam*HI−*Sal*I fragments were cloned in *Bam*HI−*Sal*I-restricted M13mp18. Mc21A−48/+46 was obtained by re-cloning the insert of Mc21B−48/+46 in the M13mp19 vector. A schematic representation of this clone is given in Figure 1.

pB21H and pU21H. pB21H was made using a pBR322 clone containing the *Eco*RI insert of Mc21A. A *Hin*dIII linker (CAAGCTTG) was inserted into the *Aoc*I restriction site at position +147 relative to the point of transcription initiation. The modified region was excised on a 1-kb *Bam*HI-fragment which was re-cloned in the *Bam*HI site of pBR322 (pB21H) or pUR222 (pU21H). Run-off transcription of these clones, digested with *Hin*dIII, gives rise to a 155-nucleotide run-off transcript.

Ml15A. M13 clone Ml15A has been described previously, but was then called Ml5A1 (Osinga *et al.*, 1982).

Construction of Bal31 deletion mutants

5′ Deletion mutants. 35 μg of *Eco*RI-linearized Mc21B−48/+46 DNA was digested with 2 U of exonuclease *Bal*31 in 100 μl at 20°C, according to the conditions specified by the manufacturer, except that the NaCl concentration was 0.2 M. At 30 s time intervals (up to 4 min), 12.5 μl aliquots were removed and added to 37.5 μl TNS (10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.1% SDS) to stop the reaction. The DNA was extracted with phenol and phenol/chloroform (1:1) and after ethanol precipitation part of the sample was used for restriction enzyme analysis to estimate the efficiency of the *Bal*31 reaction. Appropriate time samples were ligated to radiolabeled *Bam*HI linkers (CCCGGATCCGGG). After digestion with *Bam*HI and *Sal*I the digestion was run on a 12% native polyacrylamide gel and DNA fragments of desired length were eluted and used for cloning in *Bam*HI−*Sal*I-digested M13mp18. Screening for mutants was done by the Sanger dideoxy sequencing method (Sanger *et al.*, 1977).

3′ deletion mutants. 3′ deletion mutants were obtained in the same way except that Mc21B−48/+46 DNA was linearized with *Hin*dIII and that *Sal*I linkers (GGTCGACC) were used. The *Bam*HI−*Sal*I fragments were re-cloned in M13mp19

Point mutations

Point mutations were made by *in vitro* site-directed mutagenesis as described before (Osinga *et al.*, 1983). The synthetic oligonucleotides and templates used are detailed below.

Mc21A-G/T^{-2}. The 18-mer 5'-ACTTTTTACTAÄTTATAT-3' was used to mutagenize Mc21B. The insert of the resulting mutant, Mc21B-C/A was inverted to yield Mc21A−G/T^{-2}. This mutant contains the modified box sequence 5'-ATATAAT*TA-3'.

Mc21A-A/C^{-6}. The 17-mer 5'-TTATTAT*TAT*CTAAGTA-3' was used to mutagenize Mc21A. The resulting mutant, *Mc21A-A/C*$^{-6}$ has two alterations: the removal of the A at position −10 and a single A to C base substitution at position −6, yielding the mutated box sequence 5'-AT*CTAAGTA-3'.

Mc21A-T/G^{-101}. The 22-mer 5'-GATTTATAATATAAG*TAATATA-3' covering the 21S upstream region −115 to −94 was used to generate a second box in Mc21A. The mutant Mc21A-2BΔ also has a deletion from position +50 to +250 (see also Osinga *et al.*, 1983) which alters the length of run-off transcripts. *Eco*RI inserts from Mc21A-G/T^{-2} and Mc21A-A/C^{-6} were each cloned in the *Eco*RI site of pB21H to obtain plasmids (pB21H:21G/T^{-2} and pB21H:21A/C^{-6}, respectively) containing both a wild-type and a mutated 21S promoter.

mtRNA polymerase preparation

Two different procedures were used for partial purification (modified from a procedure communicated to us by J.Jaehning, University of Indiana).

Procedure I. Mitochondria were isolated after disruption of 600 g of yeast cells ('Koningsgist', a commercial *S. cerevisiae* strain, obtained from Gist-Brocades, Delft) with the use of a 'Dynomill' cell disintegrator as described (Deters *et al.*, 1976). After lysis of mitochondria using detergent (2.5% NP-40) and high salt [0.3 M $(NH_4)_2$ SO_4] and removal of debris by centrifugation (Beckmann SW27 rotor, 60 min, 20 000 r.p.m.), nucleic acids were precipitated with polymin-P. Protein was precipitated from the supernatant by addition of 0.4 g/ml $(NH_4)_2SO_4$, dissolved, dialysed and subsequently loaded on a DEAE-cellulose column (Whatman DE52) at 75 mM $(NH_4)_2SO_4$. The flow-through was immediately put on a phosphocellulose column (Whatman P11) and bound protein was eluted with 0.4 M $(NH_4)_2SO_4$. Protein was precipitated by addition of 0.4 g/ml $(NH_4)_2SO_4$ and dissolved in a small volume. After extensive dialysis it was loaded on a second phosphocellulose column (P11) which was equilibrated and eluted with a 0.15−0.6 M KCl gradient as described (Levens *et al.*, 1981a). At this stage specific initiation could be detected.

Procedure II. This was essentially carried out as described (Levens *et al.* (1981a) with the following modifications. (i) 1−4 kg of commercial yeast ('Koningsgist') was disrupted by one passage of a 30% suspension in breaking buffer through a Manton−Gaulin press operated at 7000 p.s.i. (cell breakage 40−50%). (ii) After removal of unbroken cells mitochondria were pelleted by centrifugation for 30 min at 11 000 r.p.m. in a Sorvall GSA rotor (4°C). Mitochondrial pellets were collected, resuspended, and spun down as before. Finally the resulting pellets were washed by resuspension and centrifugation for 15 min at 15 000 r.p.m. in a Sorvall SS-34 rotor. (iii) Lysis and chromatography were carried out as described (Levens *et al.*, 1981a) except for the following modifications: heparin−Sepharose CL-6B was obtained from Pharmacia instead of being home made and DEAE−Sephacel (Pharmacia) was used instead of DEAE−Sephadex A50.

Blue Sepharose chromatography

Peak fractions of the second phosphocellulose column (procedure I) were pooled, concentrated by binding to a small phosphocellulose column and step elution and, after dialysis against 20 mM potassium phosphate pH 7.8, 15% glycerol, 0.2 mM dithiothreitol, 0.05% NP-40 and 50 μM EDTA, the sample (5.6 mg protein) was loaded on a 5 ml (1.5 × 3 cm) Blue Sepharose CL-6B column (Pharmacia) equilibrated with the same buffer (P-buffer). After a wash step with P-buffer containing 0.1 M KCl (P-0.1) the column was eluted with a 40 ml gradient from P-0.1 to P-0.7. 1.2 ml fractions were collected, frozen in liquid nitrogen and stored at −70°C until use.

Transcription reactions

Plasmid DNA for *in vitro* transcription reactions was isolated by the alkaline detergent lysis method (Maniatis *et al.*, 1982). After CsCl gradient centrifugation and treatment with RNase A (50 μg/ml) and proteinase K (50 μg/ml) DNA was extracted with phenol, phenol/chloroform (1:1) and chloroform and precipitated with isopropanol. Restriction digests were extracted as above, precipitated with ethanol and dissolved in double-distilled water. Transcription reactions were carried out in 25 μl at 25°C as described (Edwards *et al.*, 1982) using 5−10 μCi [α-^{32}P]UTP (Amersham, 400 Ci/mmol) at 5 μM total UTP per reaction. In reactions containing control template, control DNA concentration was approximately equal to tester DNA concentration. Routinely 2 μl of RNA polymerase preparation was used in each reaction. For promoter identification studies active fractions from the second phosphocellulose column (procedure I) were used. Neither RNA polymerase nor DNA were present in saturating amounts under these conditions. RNA products were analysed on 6% polyacrylamide 7 M urea gels as described (Edwards *et al.*, 1982).

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