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A probable *cis*-regulatory element on yeast mitochondrial DNA responsible for cAMP-mediated transcription

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Abstract Studies from this laboratory have suggested that mitochondrial (mt) transcription in yeast (*Saccharomyces cerevisiae*) is governed by changing cellular cAMP levels, and that the mechanism of such transcriptional regulation requires cAMP-dependent protein kinase (PKA) activity; these observations, in turn, suggest a *trans*-activation process for nucleotide-dependent mt transcriptional control. Here we demonstrate a sequence-specific mtDNA-phosphorylated protein interaction, a requisite part of such a control mechanism, using filter-binding and gel mobility shift assays with mt protein extracts and mtDNA from ρ^- strains whose retained mt genes show cAMP-sensitive expression. We demonstrate that the protein-mt DNA interaction depends on PKA activity, that it specifically involves a tripartite GC-rich sequence element on yeast mtDNA, and that it does not involve mt coding or promoter sequences. Sequence analysis indicates that the GC-rich element undergoing protein interaction is present in ten copies on the yeast mt genome, and that each copy is located 5′ to a strong mt promoter; the elements appear in both orientations relative to, and at varying distances upstream from, the putatively associated mt promoter elements. The mt element shows no sequence homology to relevant nuclear *cis*-elements examined and is unrelated to published vertebrate mt *cis*-elements. Several lines of evidence and argument strongly suggest that this GC-rich element functions as the *cis*-regulatory sequence involved in cAMP-mediated transcriptional control in yeast mitochondria.

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Introduction

In eukaryotic cells, assembly of the mitochondrial (mt) respiratory system requires gene products from both the nuclear and mt genomes. While we have a good knowledge of mechanisms governing the expression of many nuclear genes specifying mt-related proteins, we have little understanding of the mechanisms controlling the local expression of mt genes (reviewed in Costanzo and Fox 1990; de Winde and Grivell 1993; Dieckmann and Staples 1994). We do, however, understand many mechanical details of the mt transcription process itself. In *Saccharomyces cerevisiae*, mt transcription proceeds from the approximately 20 promoter elements on the organellar genome; these are conserved variants of a consensus nonanucleotide sequence found 5′ to many, but not all, mt genes (e.g., Christianson and Rabinowitz 1983; see Tracy and Stern 1995 for a review). Relative RNA polymerase (RNA pol)-binding strengths have been defined for yeast mt promoters (Mueller and Getz 1986; Wettstein-Edwards et al. 1986), and sequences flanking the promoters can influence binding of the mtRNA pol (Biswas and Getz 1986). Initiation of transcription from mt promoters in yeast usually takes place at the final adenine residue.

The yeast mtRNA pol is a two-subunit enzyme distinct from all the nuclear RNA pols, but both subunits of this enzyme are encoded by nuclear genes. The yeast mt enzyme, like those of vertebrate systems, is composed of a core (catalytic) subunit and a specificity determining subunit (Winkley et al. 1985; Schinkel et al. 1986; Tracy and Stern 1995). The gene specifying the core subunit, *RPO41*, has been cloned (Kelly et al. 1987), and its DNA sequence is unrelated to those of subunits of nuclear or bacterial RNA pols (Masters et al. 1987). Two nucleus-encoded proteins affecting mt transcription have been reported in yeast (Lisowsky and Michealis 1988; Lisowsky 1990), and one of

these, the *MTF1* gene product, was shown to be the specificity subunit of the mtRNA pol (Jang and Jaehning 1991). This protein has been re-designated mtTFB, and detailed analysis of its structure and function demonstrate that it bears clear relationship to bacterial sigma factors (Shadel and Clayton 1995; Tracy and Stern 1995). Both mtTFB and Rpo41p are required for transcriptional initiation in yeast mitochondria, and the two proteins are physically associated during formation of the initiation complex; once transcription is underway, mtTFB dissociates and is re-cycled (Mangus et al. 1994). mtTFA, an abundant DNA-binding protein in yeast mitochondria, appears not to be required for organellar transcriptional initiation (Parisi et al. 1993); this protein, originally identified as the *ABF2* gene product, is an HMG-box protein and has a mammalian mt homolog (Parisi and Clayton 1991; Parisi et al. 1993; see below). Studies in vertebrate mt systems have demonstrated *cis*- and *trans*-acting elements operating in the D-loop region of the mt genome (reviewed in Clayton 1992; Shadel and Clayton 1993). The *cis* elements from several such systems have been characterized, and they are relatively long and poorly conserved in sequence. The *trans*-acting protein that binds to them has been characterized and designated as mtTFA (e.g., Parisi et al. 1993); this protein is the homolog of the yeast (sc-)mtTFA (*ABF2* gene product) referred to above. While the system(s) for activation, if any, of the mammalian mtTFA factors has (have) not been elucidated, it is clear that, unlike the situation in yeast, proper mt transcriptional initiation requires binding of the mammalian mtTFA protein to its *cis* element. A transcription termination factor has been identified and characterized in vertebrate mt systems (Daga et al. 1993).

As already mentioned, control of mt transcriptional initiation remains problematic in yeast. While *cis*-acting sequence elements have been identified in vertebrate mt systems, no similar sequences have so far been identified in the yeast organellar genome (Tracy and Stern 1995). It seems clear, however, that some mechanism does exist to govern the rate of transcriptional initiation in yeast mitochondria, other than the simple availability of the mtRNA pol holoenzyme (e.g., see Ulery et al. 1994). Previous work from our laboratory has centered on a potential role for adenosine 3′,5′ cyclic monophosphate (cAMP) in the control of yeast mt gene expression. This nucleotide is well-known to influence the expression of many genes in both prokaryotes and eukaryotes. In *Escherichia coli*, it is the mediator of catabolite repression at *lac* and other operons via the *crp* gene product, which upon binding cAMP becomes a DNA-binding protein that interacts with *cis* elements 5′ to catabolite-repressible genes (reviewed in de Crumbrugghe et al. 1984). In yeast, cAMP governs growth control and responses to nutrient limitation in the growth medium, among other processes (reviewed in Broach 1992), and it influences the transcription of some (nuclear) genes, including those specifying various r-proteins (Neuman-Silberberg et al. 1995; see also Roesler et al. 1988). However, the mechanism of cAMP-mediated transcriptional regulation is profoundly different in prokaryotes and eukaryotes. In the latter, all effects of cAMP are mediated via cAMP-dependent protein kinase (PKA); the eukaryotic cAMP-binding protein functions as the regulatory subunit of PKA, which upon nucleotide binding activates the kinase to phosphorylate one or more *trans*-acting factors; these factors then bind specific *cis*-acting DNA sequences to allow transcriptional initiation.

Many effects mediated by cAMP in eukaryotes require concomitant modulation of mt function. Studies with mutants in the *RAS* genes, which influence cAMP levels in yeast, have demonstrated that strains with constitutively high cellular cAMP levels possess giant mitochondria and enhanced rates of mt respiration (Müller and Bandlow 1987). These and other observations initially suggested to us that cAMP levels might regulate mt gene expression in yeast in some manner. We found that nutritional downshift in yeast leads to a rapid decrease in the cellular cAMP level, at least in the strains we examined, and that this attenuated cAMP level is directly responsible for the severe curtailment of mt gene expression which we and others had demonstrated to be part of the cellular response to downshift (Ray and Butow 1979a, b; McEntee et al. 1989, 1993; Cantwell et al. 1992). We then demonstrated that a *bcy1* mutant strain showed no mt transcriptional curtailment after nutritional downshift and lowered cellular cAMP levels (McEntee et al. 1993); Bcy1p is the regulatory subunit for cytoplasmic and mt PKA in yeast (Toda et al. 1987; Rahman et al. 1994; Rahman and Hudson 1995). This indicated that cAMP-responsive mt transcriptional control requires regulated PKA activity, suggesting a *trans*-activation system (McEntee et al. 1993).

The mechanism responsible for cAMP-mediated regulation of mt transcription in yeast remains to be elucidated; however, studies summarized above suggest that it operates via a *trans*-activation process similar to those known in nuclear systems. Such a mechanism would require a PKA-dependent protein-mtDNA interaction for transcriptional initiation, in turn necessitating a *cis* sequence on mtDNA. In the present study, we demonstrate a sequence-specific, phosphorylated protein-mtDNA interaction; we identify the mtDNA sequence involved in the interaction and discuss evidence suggesting that this sequence is involved in cAMP-mediated mt transcriptional control.

Materials and methods

Yeast strains, growth, manipulation of cells. Yeast strains used in the present study and their genotypes are given in Table 1. Strains were carried on YP (rich) medium (Sherman et al. 1983) with 2% glucose or 2% glycerol as the carbon source, or in minimal medium (YNB) supplemented as required with amino acids and/or nucleotides (Birky 1975), and with 2% glucose or glycerol as the carbon source. Liquid cultures for experiments were inoculated into minimal medium from fresh pre-cultures grown on the same carbon source, and growth was at 30 °C in a rotary shaking bath at 250 rpm. In some experiments, cells were subjected to nutritional downshift to insure low endogenous cAMP levels in mitochondria prepared from those cells, as previously described (McEntee et al. 1989, 1993, 1994; Cantwell et al. 1992). Cells in early mid log-phase growth ($A_{600} = 0.4–0.8$) were used for all experiments.

Preparation of mitochondria, mitochondrial DNA, and mitochondrial protein extracts. After growth as given above, cells were harvested by centrifugation, spheroplasted, and re-grown for 3 h in isotonically adjusted 1× minimal medium, as previously described (McEntee et al. 1989, 1993). Following re-growth, cultures were re-harvested, suspended in fresh, isotonically adjusted 0.05× minimal medium, and allowed to grow for 30 min, whereupon each was re-harvested; cells were lysed using glass beads and mitochondria prepared as described (Hudspeth et al. 1980). At 30 min after nutritional downshift, cells are still well within the transcriptionally selective portion of the curtailment response, and cellular cAMP levels are severely attenuated (McEntee et al. 1989, 1993; Cantwell et al. 1992). Purified mtDNAs, used in the filter-binding assays described below, were prepared as described from cells grown in YP-2% glucose (Hudspeth et al. 1980). Protein extracts for filter binding and gel mobility shift assays were obtained from mitochondria of strain IL8-8C or strain YCM2 (Table 1) by osmotic lysis as described (Müller and Bandlow 1987, 1989); extracts were dialysed extensively against a reaction buffer (see below) to remove residual endogenous cAMP. The protein concentration in all such preparations was determined by the Bradford (1976) method, using bovine serum albumin (BSA) as a standard.

Filter-binding assays. The filter-binding assay used was based on a similar system previously described by others (Keegan et al. 1986). Briefly, binding reactions were carried out in 20-µl reaction buffer (25 mM Hepes pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, 1 mM PMSF, 2 µM pepstatin A, 0.6 µM leupeptin, 50 mM KCl) in the presence/absence of 15 µM of cAMP; 1 µg of sheared salmon-sperm DNA was used as a carrier. Unless otherwise specified, each assay utilized 10 µg of mt protein extract from nutritionally downshifted cells and 25–50 fmole of ^{32}P-labeled DNA restriction endonuclease fragments from purified mtDNA from various strains or plasmids; radiolabeling of DNA fragments for use in filter-binding assays was by the standard Klenow fill-in reaction (Sambrook et al. 1989). Reaction mixtures were incubated at 30 ° for 10 min, diluted with 1 ml of the reaction buffer, and immediately passed through 26-mm nitrocellulose filter disks (0.45 µ) pre-soaked with the reaction buffer; pre-soaking buffer also contained unlabeled, sheared salmon-sperm DNA to minimize non-specific binding of radioactively labeled DNA to filter disks. Filters were washed in 1 ml of reaction buffer, air dried, and subjected to scintillation counting. In each experiment, two sets of controls were employed; one set contained no protein extract, while the other used ^{32}P-labeled restriction fragments from plasmid pBR322 to monitor non-specific binding of labeled DNA to filters. The background retained by the filters was always 10% or less of the input counts.

Gel mobility shift assays. Radiolabeled DNA probes for gel-shift assays were generated by polymerase chain reaction (PCR) amplifications that included 10 µCi each of [α-^{32}P]dATP and [α-^{32}P]dCTP (800 Ci/mmole; New England Nuclear Corp., Boston, Mass.), in a total volume of 100 µl, or else they were purified, radiolabeled clone inserts. Probes were purified by elution of labeled amplification or restriction-enzyme digestion products from 10% polyacrylamide gels run in 0.5× Tris-acetate buffer at 15 mA for 4 h. Binding reactions between labeled DNA fragments and mt protein extracts were done in a buffer containing 10 mM Hepes (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT and 10% glycerol, with or without an added 15 µM of cAMP. Binding assays were performed using input amounts of protein specified in the individual experiments shown, 10 pmole of the radiolabeled DNA fragment (usually about 10^6 dpm/reaction), and 2 µg of poly(dI-dC) in binding buffer. Reaction mixtures were incubated at 28 °C for 20 min, and samples were then electrophoresed on a 4% non-denaturing polyacrylamide gel in Tris-borate buffer (Sambrook et al. 1989). BSA was used as a control for nonspecific protein-DNA binding interactions. Visualization of radiolabeled protein-DNA complexes formed in binding reactions was by standard autoradiography using Kodak X-OMat film, or via a Molecular Dynamics phosphorimager.

Other methods. Cloning of restriction endonuclease fragments from purified mtDNAs was done by the standard method using pUC19 or pBLUESCRIPT (Stratagene Cloning Systems, La Jolla, Calif.) as a vector. Preparation of plasmid DNA for all purposes was via miniprep using either the Wizard Kit (Promega Corp., Madison, Wis.) or the cleared lysate method, followed by isopycnic centrifugation in ethidium bromide-CsCl gradients (e.g., Sambrook et al. 1989). The determination of DNA sequence was by the standard dideoxy chain-termination method on double-stranded DNA, using the Sequenase 2 system (US Biochemical Co., Cleveland, Ohio). Amplification of specific mtDNA sequences for various purposes by PCR was done in the standard manner using Ampli-*Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, Conn.) in a Thermolyne temperature cycler. Purification of PCR products for some purposes was via the Wizard PCR Prep Kit (Promega Corp.). For some studies, oligonucleotides of a specific DNA sequence and of various lengths were commercially prepared, and these were made double stranded by the Klenow reaction from a primer complementary to one end; such double-stranded oligonucleotides were labeled for use in various assays, either by the incorporation of radioactive nucleotides into the second-strand Klenow product or by random priming of the double-stranded DNA product (e.g., Sambrook et al. 1989).

Results

A cAMP-dependent mitochondrial DNA-protein binding interaction

We reasoned that if nucleotide-mediated mt transcription were governed via a *trans*-activation process, then we should be able to demonstrate a mtDNA-protein interaction which is contingent on PKA activity. To initially identify such an interaction, we used a filter-binding assay with purified mtDNA from ρ^- strain A1416 (Table 1) and mt protein extracts from nutritionally downshifted cells. The A1416 mt genome retains the *OLI1* gene plus 5′ and 3′ flanking sequences; this strain expresses *OLI1* at high level and shows severe mt transcriptional curtailment following downshift (Cantwell et al. 1992). Initial filter-binding studies, using the mix of end-labeled *Hpa*II restriction fragments from the A1416 mt genome and mt protein preparations from nutritionally-downshifted cells of ρ^+ strain IL8-8C, indicated that one fragment was retained on filters when cAMP was included in the assay mix. We then cloned all *Hpa*II fragments from A1416 mtDNA and used the filter-binding assay to identify the clone containing that *Hpa*II fragment as an insert; this clone was designated pYmA14-6. The assay results shown in Fig. 1A demonstrate that retention of linearized pYmA14-6 on filters depended on the presence of exogenous cAMP in the mt protein extract used. That is, in the absence of added cAMP, a background level of labeled plasmid was retained

Table 1 *S. cerevisiae* strains used in this study

Strain	Nuclear genotype	Mitochondrial genotype
A1416	*MAT*α *ade2 his3*	*OLI1* ρ^-
IL8-8C	*MAT*α *his3 trp1*	ρ^+
F11	*MAT*α *his3 trp1*	21S rRNA ρ^-
αQ3-3/6	*MAT*α *ade2 his3*	*CYTb, tRNA*^{glu} ρ^-
YCM2	*MAT***a** *ura3 his3 pep4::URA3*	ρ^+

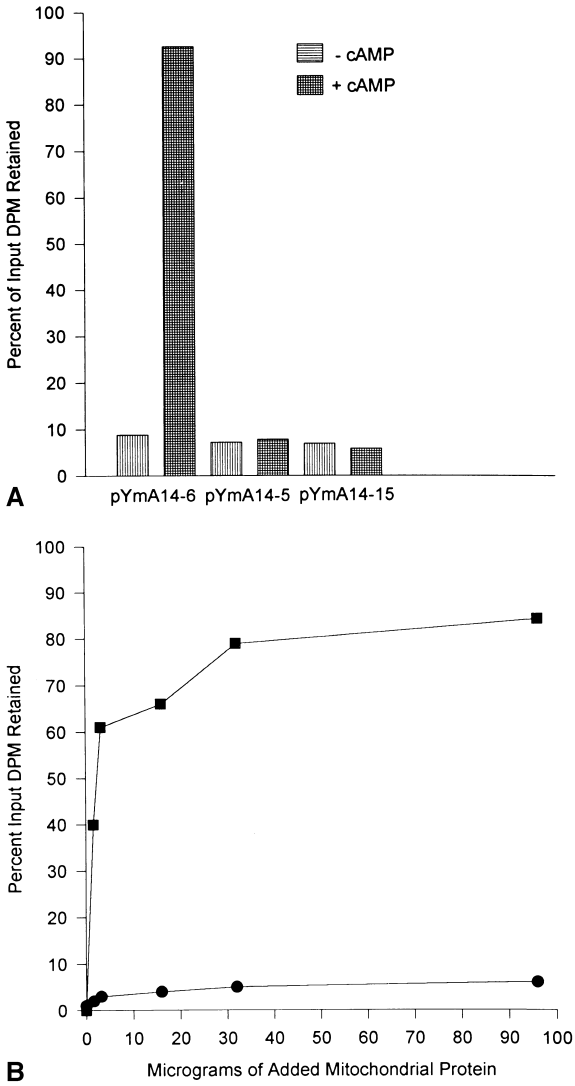

Fig. 1A, B Filter-binding assays demonstrating specificity and cAMP-dependence of interaction between the insert to clone pYmA14-6 and a PKA-phosphorylated protein. **A** filter-binding assays using the linearized plasmids pYmA14-6, whose insert contains the site of DNA-protein interaction, pYmA14-5, whose insert contains a different non-coding *Hpa*II fragment from the mt genome of strain A1416, and pYmA14-15, whose insert contains the *OLI1* coding sequence and promoter from the mt genome of strain A1416; all clones use pUC19 as a vector. The insert to pYmA14-6 is 113 bp in length; that of pYmA14-5 is 110 bp in length, and that of pYmA14-15 is approximately 550 bp in length. The protein preparation was made from purified mitochondria of nutritionally downshifted cells of strain IL8-8C (Table 1) by methods previously described (McEntee et al. 1993). Protein input into each assay was 20 μg, and the same mt protein extract was used in each assay, with or without an added 15 μM of cAMP. **B** filter-binding assays using gel-purified inserts from clone pYmA14-6 (▪) and clone pYmA14-5 (•) in the presence of increasing amounts of cAMP-treated mt protein extract. Clone inserts were prepared and radiolabeled as described in Materials and methods. The mt protein extract was prepared from cells of strain IL8-8C, and extracts included added cAMP. Each assay utilized 5 fmole of labeled DNA

on the filters whereas, when 15 μM of cAMP was included in the assay, >90% of input counts were retained. Retention was specific for the pYmA14-6 insert, since similar

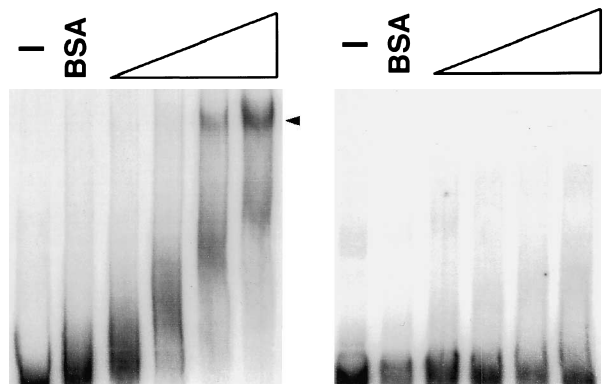

Fig. 2 Gel mobility shift assay demonstrating the production of a high-M_r product resulting from mtDNA-protein interaction between the insert to clone pYmA14-6 and a PKA-phosphorylated protein. The radiolabeled DNA fragments used in the assays were PCR products from clones pYmA14-6 (left) and pYmA14-5 (right); [^{32}P-α]-dCTP was incorporated during amplification, and each assay tube included approximately 10^6 dpm of the labeled DNA fragment. The mt protein preparation was made as described in Materials and methods from nutritionally downshifted cells of strain YCM2 and included 15 μM of cAMP; gel-shift assays were performed as described in Materials and methods. The assay tube run in the left-most lane (–) in each panel contained only labeled PCR product; the lanes marked *BSA* included that labeled DNA and 20 μg of BSA alone. Assays run in other lanes received amounts of mt protein ranging from 1–25 μg; the same mt protein preparation was used for both sets of assays. The image was produced on a Molecular Dynamics Phosphorimager. The *arrow* indicates the position of the high-M_r protein-DNA complex. Similar assays run in the absence of added cAMP produced no high-M_r protein-DNA complex

assays with other clones showed no retention of plasmid in the presence/absence of cAMP (e.g., pYmA14-5); the mtDNA-protein interaction did not involve the *OLI1* gene or its promoter, since pYm14-15, containing a *Hpa*II mtDNA fragment with both sequences as insert, was not retained. The data presented in Fig. 1B show that retention of the pYmA14-6 insert, but not that of pYmA14-5, was proportional to the input of phosphorylated mt protein in the assay. Gel-shift assays, using the pYmA14-6 and pYmA14-5 inserts with an increasing amount of cAMP-treated mt protein extract, gave a high M_r product with the pYmA14-6, but not the pYmA14-5, insert as input protein increased (Fig. 2). Thus, a DNA sequence in the pYmA14-6 insert interacts with a probable PKA-phosphorylated protein.

The DNA-protein interaction obtains with organellar genomes from other ρ^- mutants

If the DNA-protein interaction so identified is involved in cAMP-mediated mt transcriptional control, then similar interactions should occur with mtDNA fragments from ρ^- strains other than A1416 which show downshift-induced mt transcriptional curtailment. ρ^- strain F11 (Table 1) retains the 21S mt rRNA gene plus several kpb of 5′ and 3′ flanking sequence; ρ^- strain αQ3-3/6 retains the 5′-most

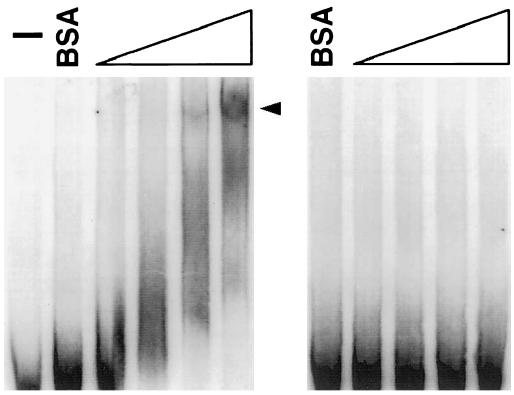

Fig. 3 Gel mobility shift assay demonstrating the production of a high-M_r product resulting from mtDNA-protein interaction between the insert to clone pYmF1146-4 and a PKA-phosphorylated protein. The labeled DNA fragments used in the assays were PCR products from clones pYmF1146-4 (left) and pYmF1146-9 (right); [^{32}P-α]-dCTP was incorporated during amplification, and each assay tube included approximately 10^6 dpm of labeled DNA fragment. The mt protein preparation was made as described in Materials and methods from nutritionally downshifted cells of strain YCM2 and included 15 μM of cAMP; gel-shift assays were performed as described in Materials and methods. The assay tube run in the left-most lane in each set of assays (–) contained labeled PCR product alone; the lanes marked *BSA* contained that DNA plus 20 μg of BSA alone. Assays run in other lanes received amounts of mt protein ranging from 1 to 25 μg; the same mt protein preparation was used for each set of assays. The image was produced on a Molecular Dynamics Phosphorimager. The *arrow* indicates the position of the high-M_r protein-DNA complex. Similar assays run in the absence of added cAMP produced no high-M_r protein-DNA complex

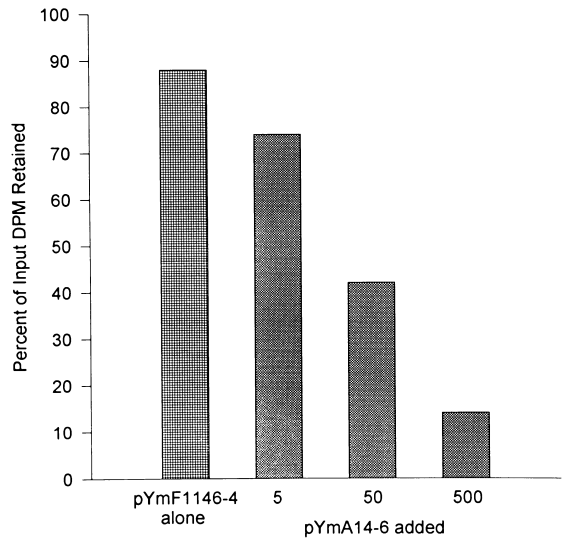

Fig. 4 Competition filter-binding assays indicating that the mtDNA insert of clone pYmA14-6 contains a DNA sequence element similar, or identical, to that within the insert of clone pYmF1146-4. Plasmids pYmA14-6 and pYmF1146-4 were linearized at an *Eco*RI restriction site in the polycloning linker region of vector pUC19 and end-labeled as given in Materials and methods. The input of labeled pYmF1146-4 in each assay tube was 10 fmole, and the input of unlabeled competitor pYmA14-6 in fmole was as specified in the figure. The mt protein extract used was from nutritionally downshifted cells of strain IL8-8C and included 15 μM of added cAMP; the input into each assay tube was 20 μg of total mt protein

portion of *CYTb*, the tRNA-glu gene upstream of it, and several kbp of upstream flanking sequence. Both mutants express their retained mt coding sequences at high level (Locker and Rabinowitz 1981; Lamb et al. 1983), and both show nutritional downshift-induced mt transcriptional curtailment (Cantwell et al. 1992). Using mtDNA from each strain, we repeated the experiments given above and cloned a fragment from each mt genome which undergoes interaction with a phosphorylated protein; the clones so identified were designated pYmF1146-4 and pYmQ3-3. As with pYmA14-6, linearized pYm F1146-4 and pYmQ3-3 were retained in filter-binding assays only when cAMP-treated mt protein extract from nutritionally downshifted cells was used, and the level of retention of each clone was dependent on the amount of phosphorylated mt protein extract employed in the assay (data not shown). Figure 3 presents a gel-shift assay using purified pYmF1146-4 insert with increasing input of cAMP-treated mt protein from nutritionally downshifted cells; as with similar experiments using the pYmA14-6 insert given above, a high M_r product appeared with increasing mt protein, indicating an interaction involving a sequence in the pYmF1146-4 insert; in contrast, the insert pYmF1146-9, the adjacent *Hpa*II fragment to that in pYmF1146-4 on the F11 mt genome, showed no formation of a high M_r product in parallel assays. Similar experiments using purified insert to clone pYmQ3-3 gave results identical to those in Figs. 1–3 (data not shown).

Prior to DNA sequence determination for the pYmA14-6, pYmF1146-4, and pYmQ3-3 inserts, we performed competition filter-binding assays using linearized plasmids in various combinations and input amounts. The results of such assays suggested that each mt-DNA fragment identified as undergoing interaction with a phosphorylated protein included a common DNA sequence. The typical assay shown in Fig. 4, in which linearized labeled pYmF1146-4 was subjected to competition for protein binding with linearized unlabeled pYmA14-6, indicated that a sequence with the latter insert effectively competed with a sequence in the former. Similar assays pairing the pYmA14-6 and pYmQ3-3 plasmids, and the pYmQ3-3 and pYmF1146-4 plasmids, gave results identical to those in Fig. 4; assays with plasmids not undergoing protein-DNA interaction showed no competition with any of the plasmids tested. Thus, several ρ^- strains which show cAMP-sensitive mt transcription include in their retained mtDNA segments a sequence undergoing cAMP-dependent interaction with an as yet unidentified protein, and these DNA sequence segments may be identical to one another.

The nucleotide sequence involved in the protein-mitochondrial DNA interaction

We determined the DNA sequences of the pYmA14-6, pYmQ3-3, and pYmF1146-4 inserts and, as expected, all

GeneBank Coordinate	Gene	Distance to Promoter	Sequence
5806	tRNAs	→1050	5'-CTCCTTC <N=0 bp> GGGGTTCGGTTCCCC <N=18 bp> GGGAGGGGTCCCTCACTCCTTCGGGGTCCC-3'
9441	*OXI1*	→ 800	5'-CTCCTCC <N=7 bp> GGGGTTCGGTCCCCC <N=20 bp> GGGAGGGGTCCCTCACTCCTTCGGGGTCCG-3'
23636	16S rRNA	→1700	5'-CTCCTTC <N=0 bp> GGGGTTCGGTCCCCC <N=20 bp> GGGAGGGGTCCCTCACTCCTTCGGGGTCCC-3'
24949	"	→ 400	5'-CTCCTTC <N=0 bp> GGGGTTCGGTCCCCC <N=8 bp> TACGGGGGTCCCTCACTCCTTCGGCCGGAC-3'
25008	"	→ 300	5'-CTCCTTC <N=7 bp> GGGGTTCGGTCCCCC <N=20 bp> GGGAGGGGTCCCTCACTCCTTCGGGGTCCG-3'
29690	*OXI3*	→ 760	5'-CTCCTTC <N=17bp> GGGGT-CGGTCCCCC <N=8 bp> AGGAGGGGTCCCTCACTCCTTCGGGGTCCG-3'
50021	*CYTb*	→3300	5'-CTCCTTC <N=0 bp> GGGATTCGGTCCCCC <N=38 bp> ACGGGGGGTCCCTCACTCCTTCGGGGTTCG-3'
63719	*OLI1*	→ 700	5'-CTCCTTC <N=7 bp> GGGGTTCGGTCCCCC <N=20 bp> GGGAGGGGTCCCTCACTCCTTCGGGGTCCG-3'
70442	21S rRNA	←4300	5'-CTCCTTC <N=0 bp> GGGGTTCGGTCCCAC <N=47 bp> CCACGGGGTCCCGCACTCCTTCGGTTCCCC-3'
74499	"	← 150	5'-CTCCTTC <N=7 bp> GGGGTTCGGTCCCCC <N=19 bp> GGGAGGGGTCCCTCACTCCTTCGGCCGGAT-3'

Fig. 5 DNA sequence of each of the ten copies of the tripartite GC-rich mt element, identified in filter-binding and gel mobility shift assays, on the yeast mt genome. At the left, the number of the initial nucleotide of the element is given according to the de Zamaroczy and Bernardi (1986) GeneBank reference sequence. Genes immediately downstream from the individual elements are indicated, along with the approximate number of nucleotides between the tripartite element and the putatively associated mt promoter for each gene; *arrows* indicate the orientation of the element relative to those promoters. *Numbers* between the three portions of the element indicate total nucleotides separating those segments in each specific copy; these spacer sequences are unconserved among copies of the element, as are the immediate 5′ and 3′ flanking sequences

sequences were relatively AT-rich. However, sequence analyses identified a three-component GC-rich element common to each insert. Comparison of this tripartite sequence element with the nearly complete yeast mt genome sequence included in GeneBank, the only such reference sequence availabe (de Zamaroczy and Bernardi 1986), showed that this element is present on the yeast mt genome in ten complete copies, although the precise DNA sequence of each element varies slightly. Figure 5 gives the DNA sequence of each copy of the element and its genomic location using coordinates from the de Zamaroczy and Bernardi GeneBank sequence for convenience; the DNA sequences separating the three portions of the element, and those flanking the elements at 5′ and 3′ end, showed no significant homology and are thus not included. Each element given in Fig. 5 is located 5′ to a strong mt promoter; the location and the mt genes controlled by each promoter are given in association with the position of each GC-rich element. Inspection of the data in Fig. 5 shows that elements are located at varying distances 5′ to their putatively associated promoters, and that they are found in both orientations in relation to these promoters (de Zamaroczy and Bernardi 1986; see also Discussion).

Protein-DNA interaction primarily involves one portion of the element

To demonstrate that interaction with a probable PKA-phosphorylated protein involved the element given in Fig. 5, and to determine whether each portion of the tripartite sequence is required for that interaction, we prepared double-stranded oligonucleotides containing the full element or portions of it and assessed the retention of these oligonucleotides in filter-binding assays (Fig. 6). Each oligonucleotide sequence was based on that of the element initially identified 5′ to *OLI1*. The largest fragment, containing the

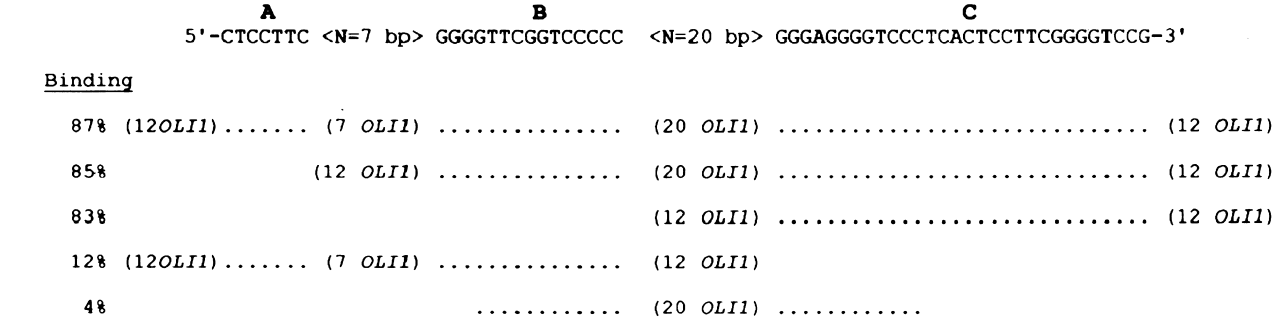

Fig. 6 Results of filter-binding assays demonstrating the interaction of the tripartite mt sequence element with a phosphorylated protein, and the contribution of the A, B, and C portions of the element to that interaction. The mt protein extract used in the assays was prepared from nutritionally downshifted organelles of strain IL8-8C and included 15 μM of added cAMP, as described in Materials and methods; 20 μg of protein extract were used in each assay. The double-stranded oligonucleotides employed were prepared and radiolabeled as given in Materials and methods, and 25-fmole fragment was used in each assay. Also, in each assay, the retention of counts on filters was compared to the retention of linearized plasmid pYmA14-6 run in a parallel assay tube at the same time and with the same protein extract; *numbers* given in the left column represent the relative binding of the specified oligonucleotide compared to that of pYmA14-6 in the parallel tube. The nucleotide sequence of the A, B, and C portions of the mt element are those of the copy of the element 5′ to *OLI1*, and that sequences is given above those of the oligonucleotides assayed; unspecified nucleotides between the three portions of the mt element, and those flanking the A and C portions, are also from the *OLI1*-derived copy but are unconserved among the ten copies of the sequence element

entire three-part element plus 5′ and 3′ flanking sequencs, was retained in the assay nearly as well as was the linearized pYmA14-6 plasmid, providing a direct demonstration that the sequence identified above is indeed involved in the protein-DNA interaction. An oligonucleotide including the A and B portions, the sequence separating those portions, and 5′ and 3′ sequences flanking them, was retained only about 10% as well as was linearized pYmA14-6. However, an oligonucleotide containing the B and C portions, and one containing only the C portion plus flanking sequence, were both retained about as well as was the oligonucleotide that included the full sequence, or the linearized pYmA14-6 plasmid. Assays using an oligonucleotide containing the 20 bp separating the B and C portions of the element, but with only small portions of those elements, was poorly retained. Thus, sequences separating the three parts of the GC-rich element are not involved in protein binding. Moreover, the primary, but probably not the sole, site of interaction appears to be the 30-bp C portion.

Discussion

Studies from this laboratory have demonstrated that transcription of mt genes in yeast is included in the overall cellular stringent response elicited by starvation for a required (marker) amino acid, and that a phenotypically identical mt transcriptional response is seen in yeast following nutritional downshift (McEntee et al. 1993, 1994). Our results further indicated that the downshift-induced curtailment of mt transcription is mediated by severely attenuated cellular cAMP levels resulting from the downshift (McEntee et al. 1993); related studies indicated that mt transcriptional curtailment following nutritional downshift, but not amino-acid starvation, requires activity of a PKA (McEntee et al. 1994). Taken together, these and other observations suggest that under some circumstances mt transcription in yeast is governed by a cAMP-dependent mechanism, and that such nucleotide-sensitive organellar gene expression is accomplished via a *trans*-activation process formally similar to that obaining for many nuclear genes in yeast and other organisms (Roessler et al. 1988; Neuman-Silberberg et al. 1995). In the present study, we provide more evidence for a cAMP-responsive mt *trans*-activation system in yeast. We demonstrate a protein-DNA interaction specific for a tripartite, GC-rich noncoding mtDNA sequence, and we show that this interaction is dependent on cAMP. Our results further indicate that the phosphorylated protein-mtDNA interaction takes place primarily at one portion of the tripartite element, and that the distribution and copy number of the entire element on the yeast mt genome are consistent with a function in cAMP-dependent mt *trans*-activation.

We initially identified the putative mt *cis* sequence as a three-part element common to the retained portion of the mt genome in three ρ^- yeast strains, each of which shows nutritional downshift-induced, nucleotide-related mt transcriptional curtailment. However, interaction between the element and the as yet unidentified PKA-phosphorylated protein(s) appears to take place primarily at the 30-bp C segment of that sequence. Computer-assisted comparison of the three-part sequence with all yeast mtDNA sequences in GeneBank confirmed that the positions given in Fig. 5 are the only ones where all three sequence segments are situated in close proximity to one another on the yeast organellar genome, and that both the relative position and sequence of the tripartite element are well conserved among different strains. Analyses for each of the three individual segments of the element, however, showed that the A portion is present at >70 positions (no mismatches), the B portion at 17 positions (four mismatches), and the C portion at 11 positions (five mismatches) on the mt genome; interestingly, the extra copy of the 30-bp C portion occurs within *CYTb* intron 3, about 450 bp 5′ to exon 4 (position about 58 000 in the de Zamaroczy and Bernardi 1986 sequence). These observations suggest that while interaction with a probable PKA-phosphorylated protein obtains primarily at the C portion of the element, transcriptional activation may require the A and B portions to be in relatively close proximity for the binding of other proteins (see below). Studies in an in vitro assay system with transcription templates of defined structure will be required to confirm this.

The yeast mt genome is known to include about 20 promoters of varying strength, but the tripartite sequence element identified here is associated with only seven of them. It is probably significant that the promoters with which the element is associated are exclusively strong ones; however, not all of the strongest promoters have an associated tripartite element. For example, of the 12 or so major transcription units in the yeast mt genome (e.g., Dieckmann and Staples 1994) those including several, but not every, tRNA gene lack the element, as does that specifying the *OX12/ORF2* coding sequences. It has generally been thought that transcriptional initiation in yeast mitochondria is globally governed, i.e., that up- or down-regulation of that process is coordinate among all mt genes such that differential transcriptional regulation does not obtain in the organelle. However, previous data from this and other laboratories have suggested that more than one mechanism exists in yeast mitochondria to govern the rate of transcriptional initiation of mt genes (e.g., Zennaro et al. 1985; McEntee et al. 1994; Ulery et al. 1994). It may well be that in the standard in vivo transcript analyses which have formed the basis for that judgement, two or more transcriptional control mechanisms operate in close concert to give the impression of global transcriptional regulation. Much more study will be required to confirm whether more than one transcriptional control mechanism operates in yeast mitochondria and, if so, what purpose(s) such multiple mechanisms might serve.

Several lines of evidence and argument suggest that the GC-rich element identified here is the *cis*-regulatory sequence required for nucleotide-mediated mt transcriptional regulation. If the tripartite mt sequence, or the 30-bp portion of it, is the *cis* element required for cAMP-

dependent transcriptional control, then the mt genomes of ρ^- strains showing cAMP-sensitive mt transcription should universally include the element, while mutants deficient in nucleotide-dependent mt gene expression should lack it. Work from this laboratory has defined several ρ^- mutants which do, and others which do not, show such transcriptional control (Cantwell et al. 1992; McEntee et al. 1993); Southern analyses of mtDNA from those mutants showing downshift-induced mt transcriptional curtailment, using an oligonucleotide probe whose sequence corresponds to the 30-bp C portion of the element, confirms that the sequence is present in each strain assayed; the sequence is, however, absent from the mtDNA of strains that do not show the downshift-induced mt transcriptional response (J.I., A.P.H., unpublished observations). Further, the distance- and orientation-independence of those elements relative to the apparently associated downstream mt promoters are consistent with the characteristics of known (nuclear) *cis*-regulatory sequences (e.g., Struhl 1989). Importantly, the data given in Fig. 5 indicate that some strong mt promoters, including that governing the transcription of *OX12*, lack a copy of the putative mt *cis* element. Interestingly, one previous study has suggested that expression of this gene may be governed differently from that of other mt genes (Zennaro et al. 1985). In addition, if the element identified in the present work is indeed responsible for nucleotide-mediated transcriptional control, then *OX12* should not show a transcriptional curtailment response to nutritional downshift, and transcript levels from it should not be effected by the high cellular cAMP levels elicited by a $RAS2^{val19}$ allele in the cell nucleus. Our initial experiments appear to confirm both predictions.

Computer analyses to determine whether the entire tripartite mt sequence element, or the 30-bp portion of it responsible for interaction with a PKA-phosphorylated protein, bears sequence homology to the *cis* element interacting with mtTFA in the D-loop of the human or mouse mt genome indicated that they do not. We also performed sequence comparisons to many other well-studied yeast and mammalian nuclear *cis* elements (e.g., AP-1, rpg/HOMOL, NFR-1), including those involved in cAMP-dependent transcriptional control (i.e., CRE), and again we were unable to identify any significant homologies. Thus, the mt element identified here appears to be unique and unrelated to other defined transcriptional regulatory DNA sequences. Interestingly, transcription of mt-related nuclear genes may be coordinately controlled with that of mt genes in mammalian cells (reviewed in Nagley 1991), and DNA sequence elements designated Mt1, Mt3, and Mt4 may be involved in this coordinate transcriptional control; the Mt3 and Mt4 elements appear to be held in common on both mammalian mtDNA and 5′ to some mt-related nuclear genes (Suzuki et al. 1990, 1991). Because evidence indicates that yeast mt promoters can function in the nucleus (Parikh et al. 1987), and because recent studies have shown that some nuclear genes in yeast are transcribed in a cAMP-dependent manner (Neuman-Silberberg et al. 1995), we asked whether the putative mt *cis*-regulatory element is present in the yeast nuclear genome. Both Southern analyses of DNA from ρ^- strains and computer analyses indicated that it is not.

As mentioned, our initial studies have indicated that expression of the mt *OX12* gene does not respond to nutritional downshift, unlike that of genes possessing the mt element 5′ to their promoters. We are now confirming and extending those initial results, and we have prepared a ρ^- strain whose mt genome retains *OX12* and its promoter for other, related experiments. Definitive evidence for a transcriptional role of the element identified and characterized here must come also from in vitro assays in which the rate of transcriptional initiation of a reporter gene can be assessed as a function of the presence/absence of the element in templates of defined structure. We have developed such an assay (Iqbal and Hudson 1996), and are currently using it to assess the transcriptional function of the intact tripartite mt element, and each of its individual parts.

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