Vol. 214, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 5, 1995 Pages 188-194

SUBSTRATES FOR YEAST MITOCHONDRIAL cAMP-DEPENDENT PROTEIN KINASE ACTIVITY

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Received August 11, 1995

We showed that transcription of mitochondrial (mt) genes in *Saccharomyces cerevisiae* is governed in part by cellular cAMP levels, and that such transcriptional control is mediated via cAMP-dependent protein kinase (cAPK) activity. Here we use *in vitro* protein kinase assays with intact mitochondria from respiring cells to define protein substrates for mt cAPK. Our data show that there are at least eight mt proteins phosphorylated in a cAMP-dependent manner, ranging in M_r from 96000 to 9500. Similar assays with organelles from an *mtf1* mutant and its wild-type parent strain show no loss of any mt cAPK target proteins, suggesting that Mtf1p (M_r=40000), the mt RNA polymerase specificity factor, does not require phosphorylation for activity. We further show, using double mutants for *TPK1*, *TPK2*, and *TPK3*, which encode catalytic subunits of the mt cAPK, that each of the eight mt substrate proteins is not phosphorylated equivalently by the individual catalytic subunits.

In *Saccharomyces cerevisiae*, cellular levels of the nucleotide adenosine 3', 5' cyclic monophosphate (cAMP) govern many critical functions, including passage through G1 of the cell cycle and responses to nutrients and nutrient limitation in the growth medium (reviewed in 1,2). The diverse pleiotropic effects caused by cAMP in yeast, and in eukaryotes in general, are universally mediated via cAMP-dependent protein phosphorylation, which in most instances modulates individual enzymatic steps in affected pathways (3). However, cAMP is known to directly influence expression of some nuclear genes, including those specifying cytoplasmic r-proteins and the mitochondrial (mt) enzyme monoamine oxidase (3). Such cAMP-dependent transcriptional regulation in eukaryotes is accomplished via phosphorylation of specific *trans*-acting protein factors, such as CREB and AP-1 (*e.g.*, 4,5). Upon phosphorylation, these proteins become DNA-binding entities specific for defined *cis*-regulatory sequences 5' to relevant genes, and binding of the *trans*-activator to its proper *cis* element allows productive transcriptional initiation. This differs from cAMP-mediated transcriptional

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0006-291X/95 $12.00

regulation in *E. coli*, which requires direct interaction of the cAMP-binding protein itself with *cis* elements 5' to catabolite-sensitive genes (reviewed in 6).

As in prokaryotes, starvation of yeast for an essential amino acid causes a rapid, severe, and coordinated curtailment of (nuclear) rDNA and r-protein gene expression, while most cellular mRNA and tRNA populations are initially far less affected (7). We and others have shown that this stringent response can also be induced in yeast via nutritional shiftdown, and that the response selectively extends beyond the nucleus to transcription of mt DNA (8,9); *i.e.*, when cells are either starved for a marker amino acid or shifted from normal to dilute medium, immediate curtailment of expression of all mt genes ensues, and the kinetics of that transcriptional curtailment are identical to those for nuclear rDNA sequences. Our studies showed that the mechanism governing stringent mt gene expression following either amino acid deprivation or nutritional downshift operates at transcriptional initiation (10). We further demonstrated that downshift of actively-growing, wild-type yeast causes a rapid decline in cellular cAMP levels, and that this fall is directly responsible for stringent mt transcriptional curtailment; we also showed that a mt cAMP-dependent protein kinase activity (cAPK) is required for nucleotide-mediated stringent mt transcriptional control (11). These and other data strongly suggest that the mechanism by which cAMP modulates mt transcription is congruent to that defined for nucleotide-dependent transcription of nuclear genes, *i.e.*, *trans*-activation.

Recent work from this group has defined the protein components required for activity of the mt cAPK. We demonstrated that this enzyme, like the cytoplasmic cAPK, utilizes the *BCY1* gene product as its regulatory subunit (12) and the *TPK1*, *TPK2*, and *TPK3* gene products as catalytic subunits (13). In the present study, we continue investigation of the cAMP-sensitive mt transcriptional control system by defining a set of protein substrates within the organelle that are phosphorylated by mt cAPK, and we provide initial evidence that, contrary to previous assertions, each of these catalytic proteins does not equivalently phosphorylate each substrate protein.

MATERIALS AND METHODS

Yeast Strains, Cell Growth. Table 1 gives yeast strains used and their genotypes. Cells were inoculated from fresh precultures into minimal medium (14) with 2% glycerol as carbon source and grown at 30°C in a rotary shaking bath at 250 rpm. Cells were harvested at early log-phase growth (A_{600}=0.3-0.6) for all experiments. Media for nutritional manipulations were properly-supplemented (1X) minimal medium, and the same medium diluted 20-fold with sterile water (0.05X; refs. 9,11).
Preparation of Mitochondria. Mitochondria were prepared and further purified as described (11,15) from cells which had been lightly spheroplasted, regrown for 3 hr in isotonically-adjusted medium to re-establish metabolism, and then shifted from 1X to 0.05X medium for 1 hr (9). Prior to the *in vitro* protein kinase or other assays, preparations of mitochondria were washed several times by resuspension in isotonic buffer using an homogenizer with Teflon pestle. Organelles were then dialyzed against a buffer containing 50 mM MOPS (pH 7.2), 50 mM NaCl, 10 mM $MgCl_2$, 50 μM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.6 M sorbitol to remove remaining endogenous cAMP. Protein concentration was determined by the Bradford method (16), using BSA as standard.

Protein Kinase Assays. *In vitro* protein kinase assays were done as described (17,18), using preparations of intact organelles and [γ-^{32}P]ATP as source of radioactive label. To reduce background phosphorylation from non-cAPK sources, organelles were preincubated in assay buffer for 5 min with 1 mM unlabeled ATP in the absence of added cAMP prior to assay; this preincubation proved more effective at background reduction than use of commercial inhibitors for non-cAPKs. Following assay, mitochondria were reharvested and lysed, and mt proteins were precipitated in cold 10% TCA; mt proteins so prepared were displayed on 8-20% gradient SDS-PAGE gels, and the gels were stained, dried, and subjected to autoradiography using Kodak X-Omat AR film.

RESULTS AND DISCUSSION

One approach to understanding the mechanism of cAMP-mediated mt transcriptional regulation at the mt promoter, or upstream of that promoter, is to identify the protein substrates of mt cAPK activity, and to determine which of them function in the mt transcriptional regulatory process. Previous work from another group identified a mt protein of M_r=40000 as the major target for mt cAPK activity in yeast (17 and see below); those assays were performed primarily in disrupted or partially disrupted organelles and were done using mitochondria from actively-growing, rather than nutritionally downshifted, cells. In order to confirm those earlier results and to try to identify additional mt cAPK substrates, we prepared mitochondria from downshifted cells of a wild-type strain (W303-1A, Table 1), and we subjected the organelles to an *in vitro* protein kinase assay in the presence/absence of added cAMP. Fig. 1 shows the results obtained in a typical mt cAPK assay. Lane 1, a control assay in which no added cAMP was present, shows the background level of non-cAPK related protein phosphorylation present in mitochondria in our assays. In Lane 2, eight proteins in the same preparation of organelles are specifically labeled when 15 μM cAMP is included in the assay mix; those proteins show M_r ranging from 9500-96000, and the intensity of labeling among them in this and other similar assays varies, presumably reflecting both the relative concentrations of each protein in mitochondria and the number of cAPK-specific phosphorylation target sites on each individual polypeptide. Lane 3 shows that labeling of the mt cAPK substrate proteins identified in Lane 2 is severely attenuated when a cAPK inhibitor is included in the assay with 15 μM cAMP.

Table 1: *Saccharomyces cerevisiae* strains used

Strain	Genotype
W303-1A	*MATα leu2 trp1 ura3 his3 ade2* ρ^+
W303LU	*MATα leu2 trp1 ura3 his3 ade2 bcy1::LEU2* ρ^+
KN79	*MATα leu2 trp1* ρ^+
KN79mtf1	*MATα leu2 trp1 mtf1::LEU2* ρ^o
TPK-1	*MATα ade8 his3 leu2 trp1 ura3 tpk2::HIS3 tpk3::TRP1* ρ^+
TPK-2	*MATα ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk3::TRP1* ρ^+
TPK-3	*MATα ade8 his3 leu2 trp1 ura3 tpk1::URA3 tpk2::HIS3* ρ^+

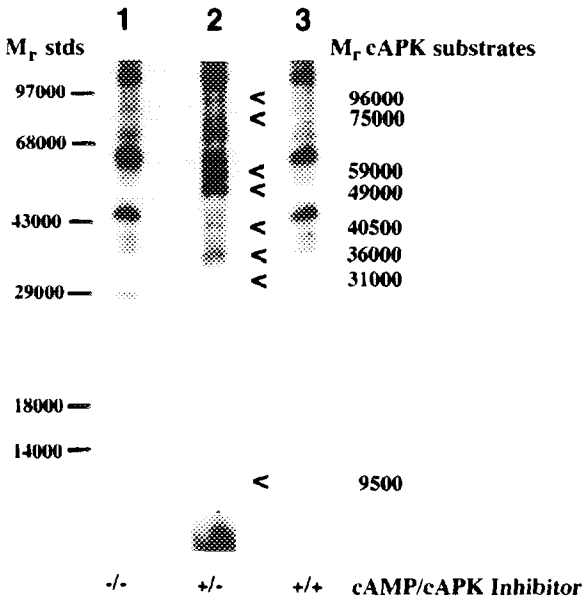

FIGURE 1.
Endogenous mt protein substrates for mt cAPK activity in *in vitro* protein kinase assays. Cells of strain W303-1A (Table 1) were grown to early log-phase in glycerol-based minimal medium and spheroplasted; following regrowth and downshift in isotonically adjusted media, cells were reharvested, broken, and mitochondria were prepared as given in Materials and Methods. *In vitro* protein kinase assays were done in intact organelles, and analysis of protein labeling was via autoradiography of dried 8-20% gradient SDS-PAGE gels. Mitochondria used in all assays shown were from the same preparation and had been preincubated with unlabeled ATP but without exogenous cAMP to lower background labeling; equal counts were loaded in each gel lane, and the autoradiograph was exposed 5 d at -70°C. **Lane 1**, labeling pattern of mt proteins in the absence of exogenously added cAMP. **Lane 2**, mt proteins labeled in the presence of added 15 μM cAMP; seven proteins are visible as mt cAPK targets and range from M_r=31000-96000; the eighth mt cAPK substrate protein, of M_r=9600, is not easily visible in this photograph. **Lane 3**, mt proteins labeled in the presence of 15 μM cAMP plus a commercially available specific cAPK inhibitor (Bethesda Research Laboratories, Gaithersburg, MD).

As mentioned, another group has identified a mt protein of M_r=40000 as the primary mt cAPK substrate (17), and we assume that the protein which runs as M_r=40500 in our gels is that same protein (see below). In that previous work, a protein of M_r=49000 was also identified as a substrate for a cAMP-independent protein kinase; in our assays, however, we routinely found a protein of the same M_r phosphorylated in a fully cAMP-dependent manner. Assays similar to those shown in Fig. 1, but comparing phosphorylation substrates in mitochondria from strain W303-1A and strain W303-LU, an otherwise isochromosomal mutant in which we had insertionally inactivated the *BCY1* gene (11), showed a lack of labeled protein at M_r=49000 in organelles from the *bcy1* mutant (data not shown). Thus the protein seen in our assays at that M_r is probably Bcy1p, the regulatory protein for yeast cAPK; this protein has a M_r=49500 and is known to act as a cAPK substrate (19).

The eight mt cAPK substrate proteins identified in the assays shown in Fig. 1 are routinely seen in such experiments in our hands, although not every substrate protein is seen in each assay. We do not fully understand why our results vary somewhat in this way from assay to assay, but the variation is probably a function of slight differences among preparations of organelles. Interestingly, the total number of mt cAPK target proteins identified in our assays is larger than that identified in previous work from another group (17). Over the course of many such assays, we have found that the overall level of cAPK activity in purified mitochondria is severely attenuated in organelles derived from spheroplasted cells which were either not allowed to regrow at all, or which had been regrown for less than 2-3 hr; moreover, in our hands mitochondria subjected to lengthy purification procedures, such as gradient centrifugation, show substantially lower overall levels of cAPK activity than do organelles prepared in the manner used in the present work. Most importantly, mitochondria from nutritionally downshifted cells have overall cellular cAMP levels which are 4-5-fold lower than those in organelles from actively-growing cells (11); presuming that turnover of both components of the mt cAPK and the mt protein targets of that enzyme is modest during the relatively brief downshift period, low organellar cAMP levels would promote vigorous phosphorylation of all appropriate mt protein substrates when cAMP is included in the assay.

The *MTF1* gene product is the specificity factor for the mt RNA polymerase (20-22). That protein is predicted to have a M_r=40000 based on the nucleotide sequence of its gene, but it runs with a slightly higher apparent Mr in some PAGE-SDS systems (22); inspection of the predicted amino acid sequence of Mtf1p suggests that one serine residue lies in a context which might serve as a cAPK target sequence (residues 70-76, ref. 21; consensus cAPK target sequence LRRASGL, ref. 23). Because cAPK-mediated phosphorylation of Mtf1p would be a straightforward means of governing its function in mt transcription, and because our protein kinase assays showed the presence of a protein of roughly the correct M_r, we assessed mt cAPK substrate proteins in a wild-type strain and in an otherwise isochromosomal mutant in which the *MTF1* gene had been insertionally inactivated (KN79, KN79mtf1, Table 1). The results of our assays clearly showed that all mt cAPK target proteins identified in Fig. 1 are present in mitochondria from both the wild-type strain and its ***mtf1*** derivative, including the protein of M_r=40500 (data not shown). Thus, at least within the limits of sensitivity of our assay, we have found no evidence that the specificity factor of the mt RNA polymerase is a cAPK substrate. Potential functions in the mt transcriptional regulatory process for the organellar cAPK target proteins other than that of M_r=49000 remain to be elucidated.

We have shown that the mt cAPK utilizes the same catalytic and regulatory subunit proteins as does its cytoplasmic counterpart (12,13). In those studies, we provided evidence that each cAPK catalytic subunit protein, Tpk1p, Tpk2p, and Tpk3p, does not function equivalently in mt transcriptional control; *i.e.*, Northern analyses suggested that mt RNA levels are lower in a strain

possessing only a functional *TPK2* gene than in equivalently grown, otherwise isochromosomal strains possessing only a functional *TPK1* or *TPK3* gene (13). We extended those results by determining whether this difference could be correlated with a specific mt cAPK substrate identified in assays such as that in Fig. 1. We prepared organelles from each of the possible *TPK* double mutant strains (strains TPK-1, TPK-2, and TPK-3; Table 1) and used those mitochondria in *in vitro* protein kinase assays to define mt cAPK substrates for each individual catalytic subunit; Table 2 summarizes the results of several such assays. Clearly, the mt cAPK substrates phosphorylated by each catalytic protein overlap extensively among the three, a result which correlates well with the observation that function among these three proteins overlaps extensively (24). However, the sets of protein substrates phosphorylated by the individual Tpk proteins are not identical; while the substrates of M_r=96000, 75000, 49000, and 31000 are equivalently phosphorylated by Tpk1p, Tpk2p, and Tpk3p, the substrate proteins of M_r=59000, 40500, 36000, and 9500 are not. Except for the M_r=49000 protein, we do not know the function of any of these mt cAPK target proteins, and the summary data given in Table 2 does not allow assignment of any particular substrate protein to a role in cAMP-mediated mt transcriptional control. Nonetheless, the data do provide a biochemical correlate to the functional nonequivalence of the three Tpk proteins previously demonstrated in our mt transcriptional studies (13). It is of interest that proteins having M_r's close to 70000 and 96000 have been reported to be associated with the mt RNA polymerase (25,26). We are now purifying our mt cAPK substrate protein of M_r=75000, in order to determine whether it functions in cAMP-sensitive regulation of mt gene expression.

Table 2: Mitochondrial protein substrates for Tpk1p, Tpk2p, and Tpk3p[1]

	Strain		
Substrate M_r	TPK-1	TPK-2	TPK-3
96000	+	+	+
75000	+	+	+
59000	-	-	+
49000	+	+	+
40500	-	-	+
36000	+	+	-
31000	+	+	+
9500	-	+	+

[1]Cells of each double mutant strain were grown to early log phase in minimal medium containing 2% glycerol as carbon source. Following spheroplasting, regrowth, and nutritional downshift in isotonically adjusted medium, cells were reharvested, broken, and mitochondria prepared as given in Methods. *In vitro* protein kinase assays were done as given in Methods, and visualization of labeling was via autoradiography of dried 12.5% polyacrylamide-SDS gels; in all assays, organelles were preincubated with unlabeled ATP without exogenously added cAMP to reduce background.

ACKNOWLEDGMENTS

We are grateful to Drs. G. Michaelis and J.R. Broach for their generous gifts of yeast strains. This work was supported by grants from the Southeastern Pennsylvania Affiliate of the American Heart Association and the Dept. Veterans Affairs Medical Research Service.

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