Short communication

Production and Characterization of Monoclonal Antibodies to Yeast Mitochondrial RNA Polymerase Specificity Factor

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Transcription of mitochondrial DNA in the yeast *S. cerevisiae* depends on recognition of a consensus nonanucleotide promoter sequence by mitochondrial RNA polymerase specificity factor, which is a 43 kDa polypeptide encoded by the nuclear *MTF1* gene. Mtf1p has only limited amino acid sequence homology to bacterial sigma factors, but functions in many ways like sigma in that it is required for promoter recognition and initiation of transcription. To analyze the core-binding region of Mtf1p, monoclonal antibodies to this protein were prepared. Recombinant Mtf1p over-produced in *E. coli* was purified to near homogeneity and used to raise monoclonal antibodies (mAbs). From fused cells screened for Mtf1p mAbs by immunodot blot analysis, 19 positive clones were initially isolated. Further analysis of positive clones by Western blotting resulted in 4 mAbs of Mtf1p.

Keywords: Mitochondrial RNA polymerase, *MTF1*, Yeast transcription.

Introduction

Yeast mitochondrial RNA polymerase is a basic apparatus for the *in vitro* transcription of mitochondrial genes. This nuclear-encoded enzyme is composed of two subunits; a core RNA polymerase and a specificity factor responsible for selective initiation at the nonanucleotide (ATATAAGTA) mitochondrial promoter (Osinga *et al.*, 1982; Winkley *et al.*, 1985; Biswas *et al.*, 1987). The core RNA polymerase is a single polypeptide encoded by the nuclear *RPO41* gene (Kelly *et al.*, 1986). Rpo41p shares nine regions of amino acids sequence similarity with the single subunit RNA polymerase of the T7 and T3

bacteriophages (Master *et al.*, 1987). These regions include the amino acids known to be required for structure and function of the catalytic domain of the phage polymerases (Delarue *et al.*, 1990; Sousa *et al.*, 1993). However, unlike the phage polymerases which function independently, Rpo41p requires a specificity factor encoded by the nuclear *MTF1* gene. This gene was initially isolated as a high-copy suppressor of a temperature-sensitive strain carrying a mutation in the *RPO41* gene (Lisowsky and Michaelis, 1988).

Mtf1p has limited amino acids sequence similarity to bacterial sigma factors (Jang and Jaehning, 1991), but functions in many ways like sigma factor in that it is required for promoter recognition and initiation of transcription. Although Mtf1p does not bind to mitochondrial promoters on its own, it interacts with the core polymerase in solution to create a holoenzyme capable of promoter recognition. Mtf1p is released after a short transcript has been synthesized and is available for interaction with a new core subunit (Mangus *et al.*, 1994). Analysis of single-point MTF1 mutations generated by a low fidelity random PCR method revealed that multiple regions of Mtf1p are involved in interacting with Rpo41p (Cliften *et al.*, 1997).

Although both Rpo41p and Mtf1p are sufficient for selective *in vitro* transcription of yeast mitochondrial genes, they solely cannot explain the *in vivo* transcriptional stimulation or repression by carbon sources. The elevated levels of Rpo41p do not increase the transcription of mitochondrial genes (Ulery *et al.*, 1994), suggesting the presence of accessory factors to mitochondrial RNA polymerase. To isolate factors and to study the interaction between Mtf1p and Rpo41p, we report here production of mAbs to Mtf1p that specifically react with the partially purified yeast Mtf1p on Western blot.

Materials and Methods

Chemicals Dulbecco's Modified Eagle Medium (DME), bovine

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calf serum, polyethylene glycol 1500 (PEG), and penicillin-streptomycin were from Gibco BRL (Gaithersburg, USA). Electrophoresis reagents were from Bio-Rad (Hercules, USA). Ampicillin, 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitrobluetetrazolium (NBT), Freund's adjuvant and all other chemicals used in this study were from Sigma Chemicals Co (St. Louis, USA).

Purification of recombinant Mtf1p Recombinant Mtf1p was induced in *E. coli* by 1 mM isopropyl-β-thiogalactopyranoside and purified according to a procedure of Lee *et al.* (1995). Briefly, Mtf1p in the insoluble fraction was resuspended in binding buffer (20 mM Tris-HCl, pH 7.9, 5% glycerol, 6 M urea, 5 mM imidazole, 500 mM KCl, and 1 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation at $39,000 \times g$ at room temperature for 20 min. The supernatant was loaded onto a 5 ml Ni^{2+}-NTA (nitriloacetic acid) chelating column equilibrated with binding buffer. Proteins nonspecifically bound to Ni^{2+}-NTA column were washed away with washing buffer (same as binding buffer with 20 mM imidazole instead of 5 mM). The recombinant Mtf1p was eluted with 1 M imidazole-containing elution buffer (same as binding buffer except for the imidazole concentration) and analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Polyacrylamide-gel electrophoresis SDS-PAGE was carried out according to the method of Laemmli (1970). For a gel, the separating gel solution containing 10% acrylamide, 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS was polymerized by adding 10 μl of TEMED and 50 μl of 10% ammonium persulfate. The separating gel was overlaid with the stacking gel solution consisting of 4.5% acrylamide, 0.125 M Tris-HCl, and 0.1% SDS. The protein sample was mixed with an equal volume of SDS-sample buffer containing 0.125 M Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.0025% bromophenol blue. For a miniature gel, 4 ml of separating gel solution and 1 ml of stacking gel solution were casted in a Mini-Protean II gel apparatus (Bio-Rad).

Production of monoclonal antibodies For injection, the purified enzyme (50 μg) was mixed with an equal volume of complete Freund's adjuvant by sonication for three 15-sec bursts at 30% maximum intensity. The antigen-adjuvant mixture was injected into a female BALB/c mouse (6–8-wk-old) intraperitoneally. The first injection was followed by three booster injections at 3 to 4-wk intervals. The final injection was given 3 or 4 d before the cell fusion without adjuvant (Ha *et al.*, 1997).

The feeder layer cells were prepared one day before fusion. A 12–18-wk-old mouse was killed by cervical dislocation, its abdominal skin carefully removed, and peritoneal cells extracted and collected by centrifugation. The cells were suspended in 60 ml HAT medium and 0.5 ml of the suspension was placed into each well of five 24-well plates. Prepared myeloma and SP2/o Ag-14 cell suspensions were combined and washed with incomplete DME by centrifugation for 3 min at $650 \times g$. The cell pellet was mixed by manually tapping the tube, and 1 ml of 50% PEG in incomplete DME was slowly added over a period of 1 min with constant swirling at 37°C. The fusion process was allowed to continue for another 90 sec at 37°C (Choi and Jeon, 1989).

About two weeks after the fusion, culture supernatants were collected and screened, first by immunodot blot analysis and then by Western blot analysis. Positive clones selected by the screening methods were transferred into 6-well plates and finally grown in tissue culture flasks (75 cm^2) and kept frozen in liquid nitrogen.

Immunodot blot Small squares (1 × 1 cm) were drawn on nitrocellulose papers and marked by numbering. One microliter of antigen solution (1 mg/ml) was applied onto each square and air-dried. The blots were incubated for 1 h in Blotto (2% nonfat dry milk in PBS), rinsed briefly with PBS, and air-dried. The blots were each cut into squares and pieces of the squares preincubated with PBS were placed in a well of 24-well culture plates containing culture fluid from hybridoma cells. The blots were processed by the procedures described in Western blot.

Western blot Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes, rinsed briefly in distilled water, and air-dried (Towbin *et al.*, 1979; Lim *et al.*, 1997). The blots were blocked with 2% nonfat dry milk in PBS for 1 h. After rinsing with PBS, the blots were incubated in culture supernatants for 1 h and washed 3 times in PBS-Tween 20 at 5 min intervals. The blots were treated with alkaline phosphatase-conjugated goat anti-mouse IgG for 1 h and washed 3 times at 5 min intervals with PBS-Tween 20. Following the final rinse for 5 min with an alkaline phosphate buffer (AP buffer, 100 mM Tris-HCl, pH 9.5, and 5 mM MgCl$_2$), the color reaction was started by incubating the blots in 10 ml of AP buffer containing 60 μl of NBT (50 mg/ml) dissolved in 70% dimethylformamide and 30 μl of BCIP (50 mg/ml) in 100% dimethylformamide. When the color reaction had developed to the desired intensity, the reaction was stopped by rinsing the membrane with several changes of distilled water.

Results and Discussion

Yeast mitochondrial RNA polymerase specificity factor was overproduced in *E. coli* and purified to near homogeneity, as shown in Fig. 1, by Ni^{+2}-charged column chromatography as previously described (Lee *et al.*, 1995). The apparent molecular weight of recombinant Mtf1p (rMtf1p) was 44 kDa, which was 1 kDa bigger in size than native Mtf1p. The size difference between recombinant and yeast Mtf1p was due to the presence of 8 histidine tags at the N-terminus of rMtf1p, which made protein purification feasible on Ni^{+2}-charged column chromatography within a single step. The authenticity of rMtf1p was confirmed by a run-off transcription reaction on a promoter containing 14S mitochondrial DNA after mixing with Rpo41p (data not shown). To enhance the immunogenicity of the protein and obtain high titration of mAbs against mitochondrial RNA polymerase specificity factor, purified Mtf1p was denatured in the presence of SDS and injected into 6–8-wk-old BALB/c mice intraperitoneally.

From two batches of fused cells, 19 positive clones were initially obtained by the immunodot blot screening method,
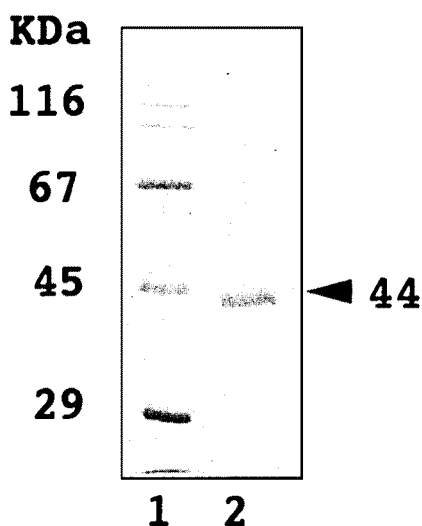
Fig. 1. Analysis of purified recombinant Mtf1p by SDS-polyacrylamide gel electrophoresis. Mtf1p purified through Ni^{+2}-NTA column chromatography appeared as a single protein with a molecular weight of 44 kDa. Proteins were visualized with Coomassie Brilliant Blue staining.

Fig. 2. Immunodot blot screening of monoclonal antibody to Mtf1p. One microgram of antigen was spotted onto nitrocellulose and processed for immunoreactive reactions with culture fluid from the indicated hybridoma cell. Spots were visualized by the alkaline-phosphatase reaction with BCIP and NBT. Each individual hybridoma cell was numbered.

as shown in Fig. 2. Hybridoma cells numbered 32, 83, 97, and 136 showed strong immunogenic reactions in the dot blot analysis, whereas the other cells reacted weakly with antigen. The hybridoma cells from positive wells were transferred onto 6-well plates and monoclonal antibody producing cells were isolated by limiting dilutions. To check the specificity of the mAbs, yeast whole cell extracts were prepared as previously described (Jang and Jaehning, 1991) and separated on 10% gel by SDS-PAGE, transferred onto nitrocellulose, probed with each selected clone, and visualized by the alkaline-phosphatase reaction with BCIP and NBT.

A single immunoreactive band of 4 mAbs with yeast whole cell extracts was detected on Western blotting, as shown in Fig. 3. Hybridoma cells producing monoclonal antibodies MT83 and MT97 contained the highest immunogenic titer among clones tested, suggesting that hybridomas producing these antibodies contained stable antigenic recognition sites. However, hybridomas producing mAbs MT32 and MT136 showed weak immunogenic reaction on Western blot analysis, suggesting that they either produced unstable antibody or progressively lost antibody production ability during cell generations. As shown in immunodot blot analysis, the other hybridoma cells produced undetectable mAbs on Western blot analysis and were thus discarded. Because goat anti-mouse IgG antibody was used as a second antibody, all mAbs screened in this study are IgG classes.

Considering the immunogenic intensities of mAbs with Mtf1p on filters, at least three different mAbs that may have their own specific epitopes were isolated and their epitope-mappings are being undertaken. The mAbs
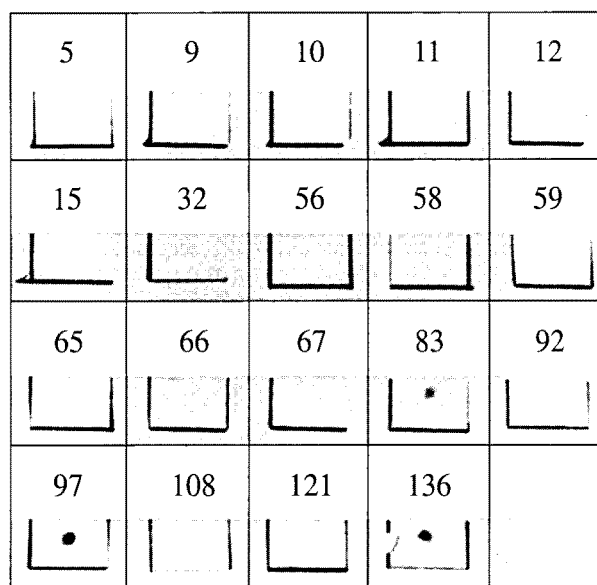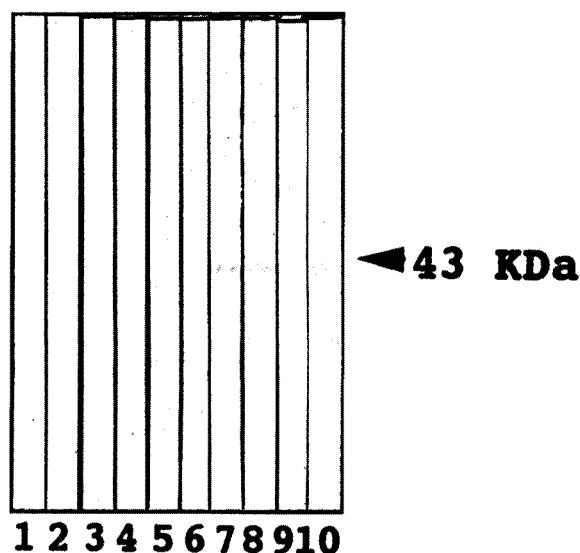
Fig. 3. Western blots probed with representative mAbs to partially purified yeast Mtf1p. The yeast whole cell extracts were prepared by breaking cells with a glass-beater. The whole cell extract was fractionated on DEAE and phosphocellulose column chromatography. Fractions (10 µg) containing Mtf1p activity were separated on 10% polyacrylamide gel, blotted onto nitrocellulose papers and probed with individual mAbs indicated as follow: 1, mAb MT9; 2, mAb MT11; 3, mAb MT12; 4, mAb MT32; 5, mAb MT56; 6, mAb MT66; 7, mAb MT83; 8, mAb MT97; 9, mAb MT108; 10, mAb MT136.

obtained in this study can be used to study the structure-functions of Mtf1p, such as its core polymerase binding sites, and to isolate Mtf1p-associated factors.

References

Biswas, T. K., Ticho, B. and Getz, G. S. (1987) *In vitro* characterization of the yeast mitochondrial promoter using single-base substitution mutants. *J. Biol. Chem.* **262**, 13690–13696.

Byung, J. H., Suk, J. K., Ji, S. P., Ree, A. Y., Dong, E. L., Ook, J. Y., Koo. W., Hyun, S. K. and Myung, S. O. (1997) Monoclonal antibody CFC-6, which binds to helix II, inhibits erythropoietin-induced bioactivity. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **30**, 315–319.

Chang, S. L., Jeong, S. C., Soo, K. S., Kyu, C. L. and Chan, H. L. (1997) Transforming growth factor-β enhances tyrosine phosphorylaton of two cellular proteins in HEL cells. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **30**, 119–124.

Choi, E. Y. and Jeon, K. W. (1989) A spectrin-like protein present on membranes of *Amoeba proteus* as studied with monoclonal antibodies. *Exp. Cell Res.* **185**, 154–165.

Cliften, P. F., Park, J. Y., Davis, B. P., Jang, S. H. and Jaehning, J. A. (1997) Identification of three regions essential for interaction between a σ-like factor and core RNA polymerase. *Genes & Dev.* **11**, 2897–290.

Delarue, M., Poch, O., Tordo, N., Moras, D. and Argos, P. (1990) An attempt to unify the structure of polymerases. *Protein Eng.* **6**, 461–467.

Jang, S. H. and Jaehning, J. A. (1991) The yeast mitochondrial

RNA polymerase specificity factor, *MTF1*, is similar to bacterial σ factors. *J. Biol. Chem.* **266**, 22671–22677.

Kelly, J. L., Greenleaf, A. L. and Lehman, I. R. (1986) Isolation of the nuclear gene encoding a subunit of the yeast mitochondrial RNA polymerase. *J. Biol. Chem.* **261**, 10348–10351.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lee, E. A., Yang, J. S. and Jang, S. H. (1995) A single-step purification of the *Saccharomyces cerevisiae* mitochondrial RNA polymerase specificity factor overproduced in *Escherichia coli*. *Mol. Cells* **5**, 217–223.

Lisowsky, T. and Michaelis, G. (1988) A nuclear gene essential for mitochondrial replication suppresses a defect of mitochondrial transcription in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **214**, 218–233.

Mangus, D. A., Jang, S. H. and Jaehning, J. A. (1994) Release of the yeast mitochondrial RNA polymerase specificity factors from transcription complexes. *J. Biol. Chem.* **269**, 26568–26574.

Master, B. S., Stohl, L. L. and Clayton, D. A. (1987) Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell* **51**, 89–99.

Osinga, K. A., De Haan, M., Christianson, T. and Tabak, H. F. (1982) A nonanucleotide sequence involved in promotion of ribosomal RNA synthesis and RNA priming of DNA replication in yeast mitochondria. *Nucleic Acids Res.* **10**, 7993–8006.

Sousa, R., Chung, Y., Rose, J. and Wang, B. (1993) Crystal structure of bacteriophage T7 RNA polymerase at 3.3 Å resolution. *Nature* **364**, 593–599.

Towbin, H., Straehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.

Winkley, C. S., Keller, M. J. and Jaehning, J. A. (1985) A multicomponent mitochondrial RNA polymerase from *S. cerevisiae*. *J. Biol. Chem.* **260**, 14214–14223.