cDNA Cloning of Human Mitochondrial Transcription Factor 1 with Polymerase Chain Reaction and Overexpression of the Factor in *Escherichia coli*

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ABSTRACT

A human cDNA encoding mitochondrial transcription factor 1 (mtTF1) was isolated by screening a HeLa cell cDNA library by using polymerase chain reaction. In the present experiment, mtTF1 cDNA clone could be obtained more rapidly, easily and at a low cost as compared with conventional cloning techniques. The size of the cDNA fragent was approximately 2 kb, indicating that the cDNA is almost full length. For the mass production of mtTF1 as a fused protein, the cDNA fragment was inserted into an expression vector pUC19. The recombinant plasmid encodes the mature mtTF1 protein and extra 20 amino acids at its amino terminus derived from *Lac Z'* in the vector and signal peptide of mtTF1. The fused protein of 26 kDa was overproduced in *Escherichia coli,* and had the same characteristics of DNA binding as natural mtTF1.

INTRODUCTION

Mitochondrial transcription factor 1 (mtTF1) is the only accessory protein required for the transcription initiation of human mitochondrial DNA (mtDNA) by mitochondrial RNA polymerase[1]. The mtTF1 binds to the upstream regulatory elements of transcriptional promoters in the mtDNA and initiates accurate and efficient transcription from the sites[2]. Recently Parisi and Clayton have cloned the mtTF1 cDNA[3] and showed, by analyzing the protein sequence predicted from the cDNA, that mtTF1 is a family of high-mobility group proteins. To clarify the function of mtTF1 in the mitochondrial transcription, a large amount of purified mtTF1 should be required. We therefore attempted to mass-produce mtTF1 in *Escherichia coli.*

In cDNA cloning, construction and screening of the library is generally quite time-

consuming and laborious experiment and is more expensive in the case using nonradioactive procedures. Recently polymerase chain reaction (PCR) is becoming to be applied to various DNA technologies because of its high sensitivity, specificity, and simplicity[4-6]. In this study we attempted to apply PCR to cDNA cloning of mtTF1. The phages of HeLa cell cDNA library were divided into 120 groups, and positive groups containing mtTF1 cDNA were found by PCR using primers derived from known mtTF1 cDNA sequence. The mtTF1 cDNA of full length was finally isolated from the positive groups by plaque hybridization, and was subcloned in an expression vector. Recombinant (r-) mtTF1 with native DNA binding activity was thereby overexpressed in *E. coli*.

MATERIALS AND METHODS

Materials. A cDNA library of HeLa cell constructed in λgt11 was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). The library contains approximately 1.5×10^6 independent clones and has an average insert size of 1.6 kb (range: 0.6 to < 4.0 kb). Oligonucleotide primers summarized in Table 1 were synthesized on an Applied Biosystems Model 381 A DNA synthesizer (Forster City, CA) and purified with OPC column. Plasmid vectors, pUC18 and pUC19, and *Tth* DNA polymerase were obtained from TOYOBO Co., LTD. (Osaka, Japan). Restriction endonucleases were obtained from TAKARA SHUZO Co., LTD. (Kyoto, Japan).

PCR [4-6]. PCR was conducted in a programmable thermal controller (MJ Research, Inc., Model PTC-150, Watertown, MA). The cDNA regions in recombinant phages were amplified by PCR in a reaction mixture (20 μl) containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatin, 0.5 μM λgt11-f primer, 0.5 μM λgt11-r primer, 0.6 mM dATP, dGTP, dCTP, TTP each, and 200 ng of DNA. The mixture was incubate for 5 min at 94℃ and subjected to 20 cycles of PCR with the following cycle profile: 1 min at 94℃, 1 min at 55℃, and 2 min at 72℃. At the end of the last cycle, an extension step, 7 min at 72℃, was run. PCR for amplification of target sequence was conducted in a mixture (20 μl) containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatin, 1 μM TF1-f primer, 1 μM TF1-r primer, 0.6 mM dATP, dGTP, dCTP, TTP each, and 5 μl of PCR mixture of the previous amplification. The thermal program was as follows: 5 min at 94℃; 10 cycles of 1 min at 94℃, 1 min at 55℃, and

Table 1 Sequences of the oligonucleotide primers used in this study.

Name	Sequence	Reference
TF1-f	5′-CCTCGCTAGTGGCGGGCATG-3′	(3)
TF2-f	5′-ATGGCGTTTCTCCGAAGCAT-3′	(3)
TF1-r	5′-TTGTGAACACATCTCAATCT-3′	(3)
λgtll-f	5′-GGTGGCGACGACTCCTGGAGCCCG-3′	(17)
λgtll-r	5′-TTGACACCAGACCAACTGGTAATG-3′	(17)
LSP-f	5′-ACAAAGAACCCTAACACCAG-3′	(18)
LSP-r	5′-AGGGGAAAATAATGTGTTAG-3′	(18)

1 min at 72℃; 10 cycles of 0.5 min at 94℃, 1 min at 55℃, and 1 min at 72℃; 10 cycles of 0.5 min at 94℃, 1 min at 55℃, and 2 min at 72℃; 10 cycles of 0.5 min at 94℃, 0.5 min at 55℃, and 3 min at 72℃; and 7 min at 72℃. The PCR products were analyzed by agarose gel electrophoresis in 1×TBE (90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA).

Plaque hybridization. Unless otherwise noted, manipulations of the phage and plaque hybridization were conducted as described in the instruction manual of CLONTECH. The probe DNA for the hybridization was a PCR product of 900 bp obtained by using TF1-f and TF1-r primers. The fragment was cloned into *Sma*I site of pUC18, excised by *Eco*RI- and *Hin*dIII-digestion from the plasmid, and labeled with digoxigenin-11-dUTP (Boeringer Mannheim GmbH, Mannheim, Germany) by random priming method. The digoxigenin probe was detected by DIG Luminescent Detection Kit (Boeringer Mannheim GmbH).

DNA binding analyses. E. coli JM105 transformed by pmtTF1ex, a mtTF1 expression plasmid, was grown in LB medium containing 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37℃ overnight. The soluble protein was extracted from the cells according to the method of Marston[7]. After centrifugation at 12,000 rpm with HITACHI RT15A2 rotor for 10 min, the supernatant was quickly frozen and stored at −80℃. Light strand promoter (LSP) of human mtDNA was chosen for r-mtTF1 binding analysis[8]. The DNA containing LSP region was amplified from a human DNA by using LSP-f and LSP-r primers, and cloned into *Sma*I site of pUC18. An 125 bp of inserted fragment excised by *Eco*RI- and *Bam*HI- digestion was labeled at its 5′ termini with digoxigenin-11-dUTP and Klenow fragment of *E. coli* DNA polymerase I. Southwestern blotting was conducted as follows[9]. *E. coli* extract was electrophoresed on a 12.5% sodium dodecyl sulfate- (SDS-) polyacrylamide gel[10] and transferred to PVDF membrane (Millipore Corporation, Bedford, MA) with semidry electroblotter (ATTO, AE-6675, Tokyo, Japan) in a blotting buffer consisting of 20 mM Tris-base, 25 mM glycine, and 20% methanol at 100 mA constant for 2 hrs. The filter was first blocked with 5% (w/v) nonfat dry milk in buffer A (10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 10 mM NaCl, and 7 mM β-mercaptoethanol) at room temperature for 1 hr, and then incubated in buffer A containing 0.25% of nonfat dry milk and 45 ng of digoxigenin-labeled LSP probe for another hour. The filter was washed 4 times with buffer A for each 5 min and baked at 80℃ for 30 min. Digoxigenin probe was detected by DIG Luminescent Detection Kit . Gel mobility shift assay was conducted essentially as previously described[11]. One microlitter of bacterial extract was incubated in a 12 μl of buffer B (10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 10 mM NaCl, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 100 μg/ml bovine serum albumin , and 12% glycerol) containing 2 μg sheared salmon sperm DNA at 10 min on ice. After adding 1 ng of the digoxigenin-labeled LSP probe, the mixture was incubated for 20 min on ice, and then loaded on 5% polyacrylamide gel in 0.5×TBE. The DNA-protein complex was electrophoresed at 10 mA constant until xylen cyanol reached at 3 cm above the bottom of the gel, and transferred to Hybond-N membrane (Amersham Inc., Arlington

Heights, IL) with a semidry electroblotter in 0.5×TBE at 50 mA constant for 1 hr. After baking the membrane, the digoxigenin probe was detected by DIG Luminescent Detection Kit.

RESULTS AND DISCUSSION

Cloning of mtTF1 cDNA by screening of cDNA library using PCR. A cloning strategy of mtTF1 cDNA was schematically shown in Fig. 1. HeLa cell cDNA library containing 1.5×10^6 independent clones was plated on 120 dishes at a density of 2×10^4 plaques per dish. Phage particles were collected from each dish in a 3-ml phage dilution buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.01% gelatin), and

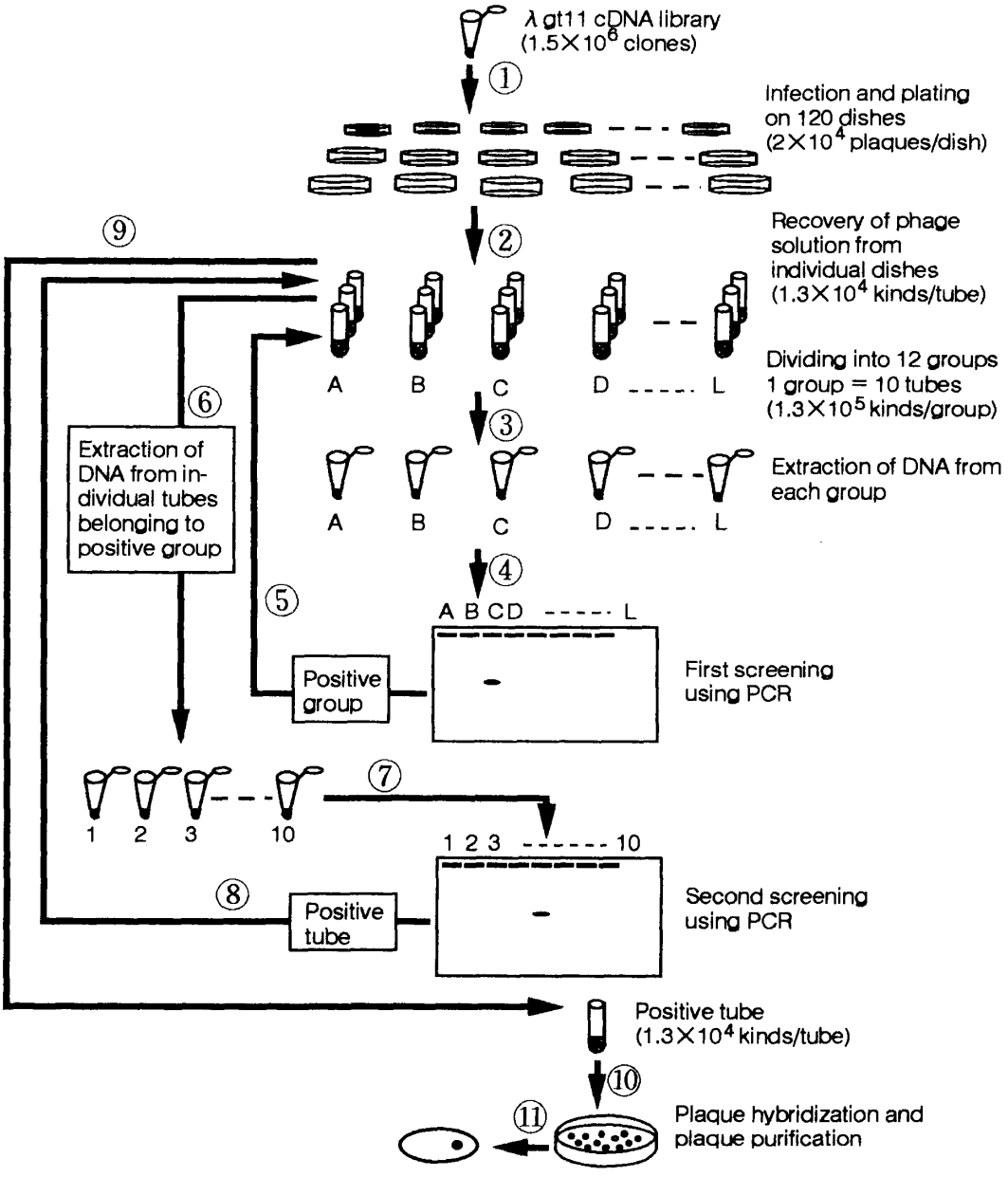

Fig. 1 *Cloning strategy of mtTF1 cDNA.*

stored in individual tubes. The phage solution (a 50-μl aliquot from each tube) was pooled every 10 tubes as one group so that the phages of 120 tubes were divided into 12 groups (A to L). One group, therefore, contains about 1.3×10^5 kinds of phages. The phage DNA of each group was extracted[12] and used as a template DNA of PCR for first screening. *Tth* DNA polymerase, instead of *Taq* DNA polymerase, was employed for amplification because of its strong activity even for crude DNA template[13]. When mtTF1 cDNA was directly amplified from the library DNAs using the mtTF1 primers, many nonspecific bands appeared. To avoid random priming on the vector sequences, cDNA region of the phage DNAs were first amplified by using λgt11 primers and the amplification of the target sequence was then achieved. The result of the first screening was shown in Fig. 2A. A 900-bp fragment, as expected from the mtTF1 cDNA sequence, was generated from nine groups (A, B, C, F, G, I, J, K, and L). One of positive groups, group A, was chosen for the second PCR screening. Phage solution in the original 10 tubes belonging to group A (namely subgroup A-1 to A-10, containing about

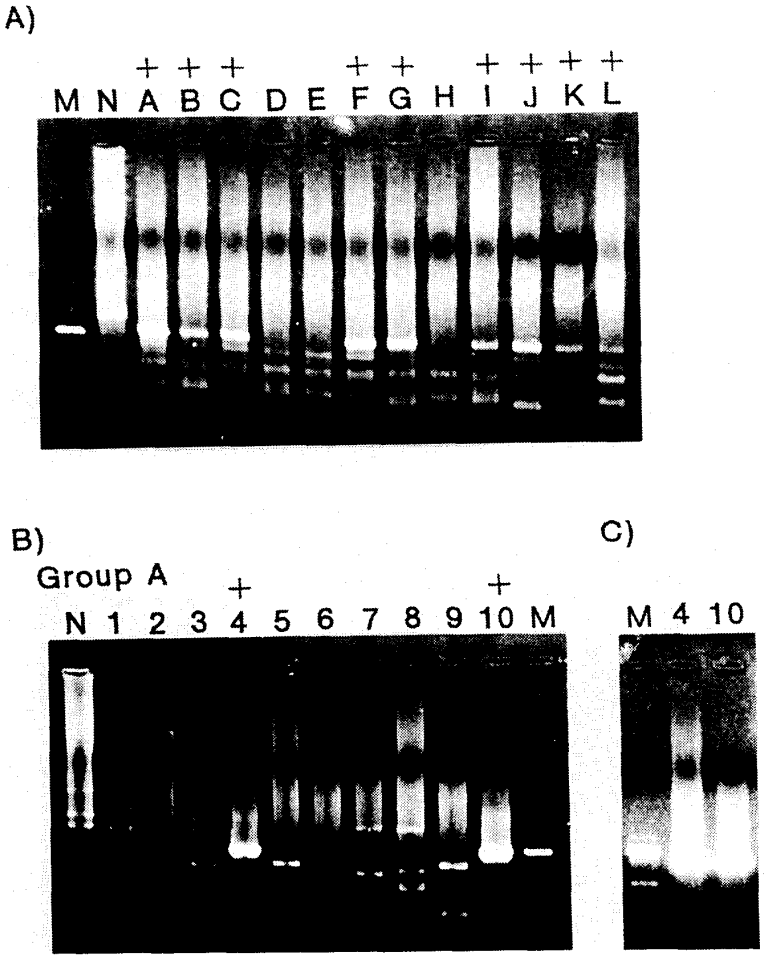

Fig. 2 *PCR screening.* A) First PCR screening. Lane M is a molecular weight maker of 900 bp. Lane N indicates a negative control by using λgt11 vector as a template. Lanes A to L indicate the phage groups from the library. The positive groups were marked "+" above the lanes. B) Second PCR screening. Lanes 1 to 10 indicate subgroups belongs to group "A". C) Nested PCR. DNA from subgroup A-4 or A-10 was used as a template.

1.3×10^4 kinds of phages in each tube) was individually taken out from the tube and DNA from each tube was extracted. The subgroup containing mtTF1 cDNA was sought by the same procedure as the first screening. A 900-bp fragment was generated from subgroups A-4 and A-10, indicating that the subgroups contained mtTF1 cDNA (Fig. 2B). Accurate amplification of mtTF1 cDNA was confirmed by nested PCR[14] using a TF2-f primer, instead of TF1-f, that is complementary to an internal sequence of mtTF1 cDNA (Fig. 2C). A product of 768 bp perfectly coincides with the expectation. The PCR screening remarkably reduced the number of the candidates to be screened from 1.5×10^6 to 1.3×10^4 without time-consuming and trouble procedures.

The phages of subgroup A-4 were plated on a 90-mm dish and then nonradioactive plaque hybridization was carried out using the 900-bp PCR product as a probe. Several weak signals were obtained (Fig. 3A). The positive plaques were picked up, and subsequently purified by second hybridization. One of the positives had 2.0 kb insert fragment and was thought to contain full-length cDNA of mtTF1, because it is the same in size as mRNA of mtTF1 (Fig. 3B).

Construction of mtTF1 expression plasmid. Construction of mtTF1 expression plasmid was schematically shown in Fig. 4. pUC19 was chosen as an expression vector in *E. coli* system, because it has a strong and inducible promoter of *lac* Z and available polycloning sites[15]. The mtTF1 cDNA fragment was excised from λgt11 vector by *Eco*RI digestion and inserted into *Eco*RI site in pUC19. To remove a signal peptide sequence of mtTF1 as much as possible and to coincide the reading flame of mtTF1 cDNA with that of *Lac Z* , the plasmid was processed as described as follows. The recombinant plasmid was cleaved with *Hin*dIII and *Sal*I and the small fragment was removed by agarose gel electrophoresis. Sticky ends of the plasmid were trimmed by S1 nuclease digestion. The resulting DNA was circularized by T4 DNA ligase and was

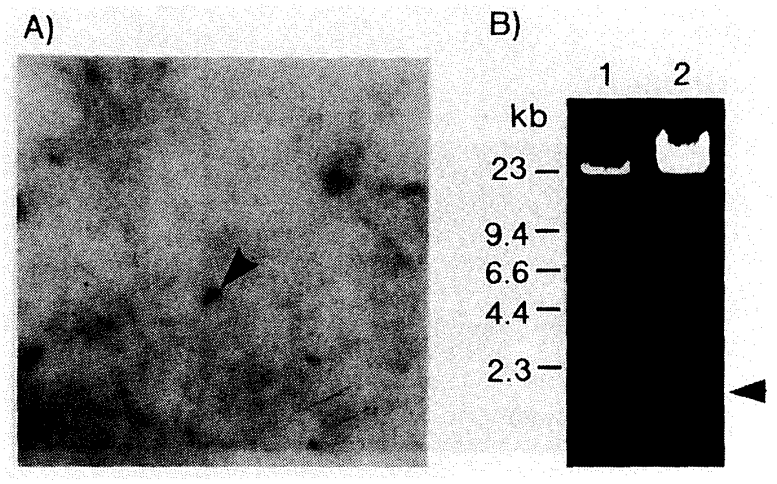

Fig. 3 *Isolation of mtTF1 cDNA.* A) Plaque hybridization of subgroup A-4. Arrow head showed a positive signal. B) A cDNA fragment of mtTF1. Lane 1 is *Hin*dIII-digested λ DNA as a molecular marker. The purified phage DNA having mtTF1 cDNA was digested with *Eco*RI and electrophoresed on 1% agarose gel (lane 2). Arrow head showed mtTF1 cDNA fragment.

named pmtTF1ex. The DNA sequence predicts that pmtTF1ex encodes a 26-kDa fused protein having matured mtTF1 and extra 20 amino acid in its amino terminal derived from *Lac Z* and remaining signal peptide.

Expression of recombinant mtTF1. pmtTF1ex was introduced into *E. coli* JM105. The cells were grown overnight and cracked by a sampling buffer of SDS-polyacrylamide gel electrophoresis (PAGE)[10]. Extract was subsequently analyzed by SDS-PAGE and coomassie brilliant blue (CBB) staining. As shown in Fig. 5A, the clone containing the pmtTF1ex had a new band at about 26 kDa. The size of the 26-kDa protein agrees well with the value predicted from the expected sequence.

We tested that the 26-kDa protein binds to mitochondrial promoter sequence. Proteins of the recombinant were electrophoresed in SDS-polyacrilamide gel, and blotted on a nylon membrane. After renaturation treatment the 26-kDa protein specifically bound to LSP probe DNA (Fig. 5B). Gel mobility shift assay using a soluble bacterial extract and LSP probe DNA showed that only the extract of the clone having pmtTF1ex formed a protein-DNA complex (Fig. 5C). Competition experiment with cold LSP DNA confirmed the specificity of the complex formation. The results indicated that the 26-kDa protein is r-mtTF1 and the protein has the same property in DNA binding as natural mtTF1. The r-mtTF1 could be available for further study of mtTF1 characterization[16].

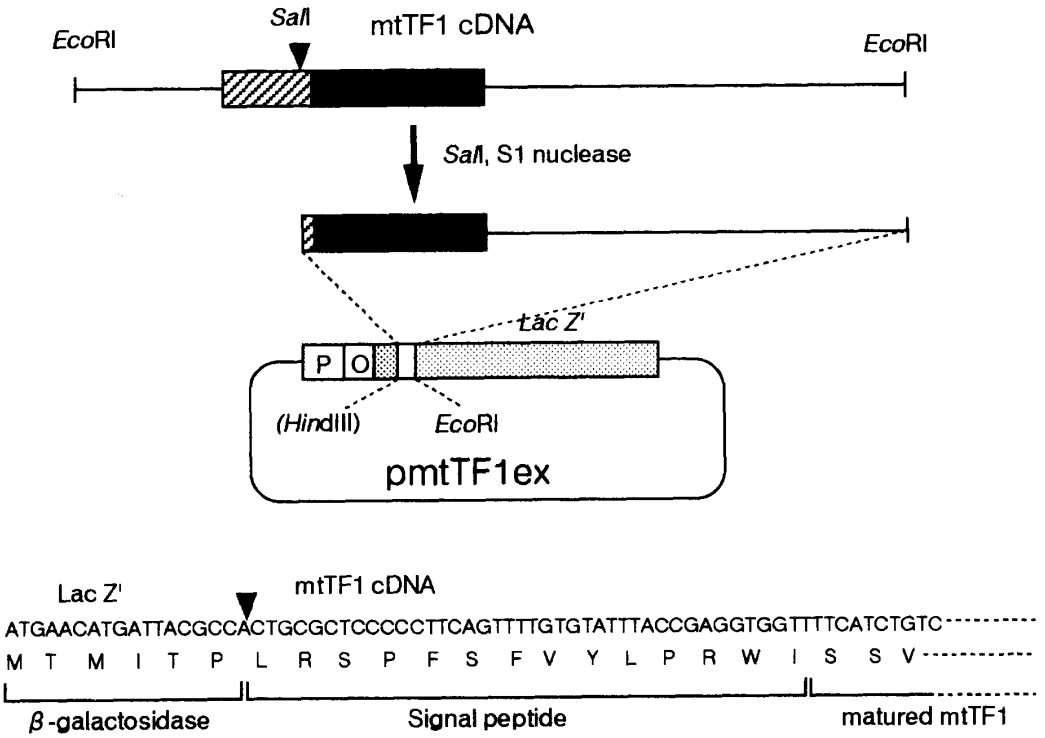

Fig. 4 *Construction of expression plasmid pmtTF1ex.* Upper) Solid box, slashed box, and dotted box indicate the region of matured mtTF1, signal peptide of mtTF1, and *Lac Z'*, respectively. Lower) Predicted nucleotide sequence and amino acid sequence around the junction. DNA sequences of pUC19 and mtTF1 cDNA are cited from references 15 and 3, respectively.

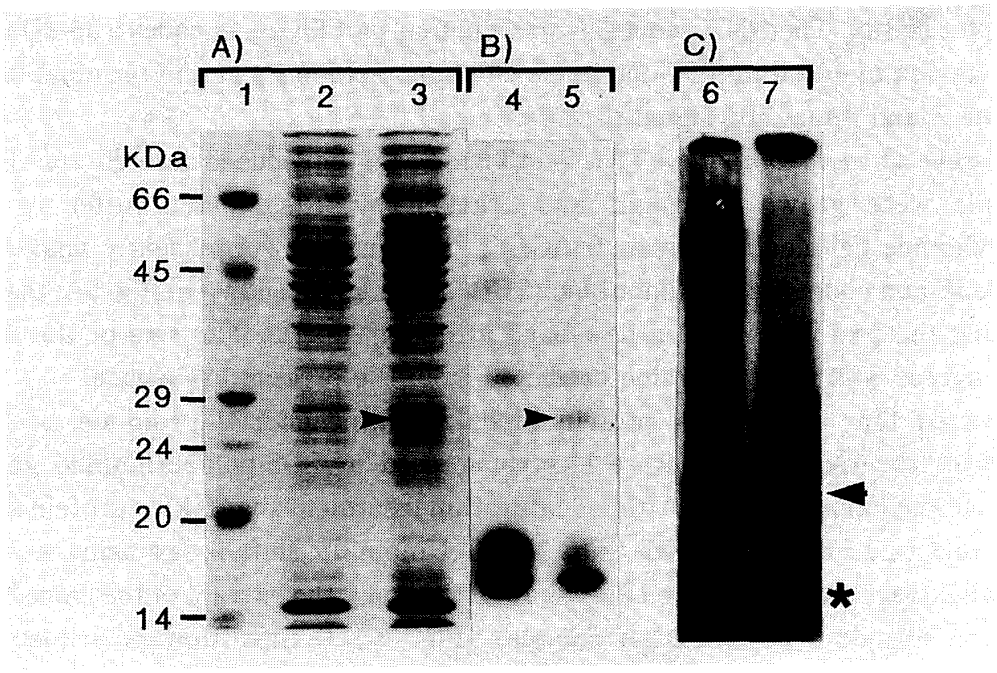

Fig. 5 *Expression of r-mtTF1.* A) SDS-PAGE and CBB staining of proteins in *E. coli* carring pmtTf1ex. Lane 1 is molecular weight makers (Sigma Chemical company, St. Louis, MO, MW-SDS-70L kit). The whole cell extract from JM105 carring pUC19 (lane 2) or pmtTF1ex (lane 3) was electrophoresed on 12.5% SDS-polyacrylamide gel[19], and stained with CBB. Additional band corresponding with r-mtTF1 was indicated by an arrow head. B) Southwestern blotting. The proteins in the same gel as A were transferred to the PVDF membrane and renatured. The membrane was then incubated with LSP probe described as Materials and Methods. Lane 4 is the extract from JM105 carring pUC19, and lane 5 is from pmtTF1ex. Arrow head indicates the binding of r-mtTF1 to LSP probe. C) Gel mobility shift assay. The cell extract from JM105 carring pUC19 (lane 6) or from pmtTF1ex (lane7) was incubated with LSP probe, and the electrophoresed on the native gel. A star is free probe. Band shift due to the binding of r-mtTF1 to LSP probe is indicated by an arrow head.

REFERENCES

1) G.S. Shadel and D.A. Clayton : Mitochondrial transcription initiation. Variation and conservation, J. Biol. Chem. **268**, 16083—16086 (1993).

2) R.P. Fisher and D.A. Clayton : Purification and characterization of human mitochondrial transcription factor 1, Mol. Cell. Biol. **8**, 3496—3509 (1988).

3) M.A. Parisi and D.A. Clayton : Similarity of human mitochondrial transcription factor 1 to high mobility group proteins, Science **252**, 965—969(1991).

4) H.A. Erlich : PCR Technology, Principles and applicatins for DNA amplification, Stockton Press, New York, NY (1989).

5) M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White : PCR protocols, a guide to methods and applications, Academic Press, INC., San Diego, CA (1990).

6) J. Ellingboe and U.B. Gyllensten : The PCR Technique, Eaton Publishing, NATIC, MA (1992).

7) F.A.O. Marston : The purification of eukariotic polypeptides expressed in *Escherichia coli.* in DNA Cloning, pp. 59—88, IRL Press, Oxford, UK (1987).

8) .D. Chang and D.A. Clayton : Precise identification of individual promoters for transcription of

each strand of human mitochondrial DNA, Cell **36**, 635—643 (1984).

9) S. Cooley, C. Weelter and N. Blin : Nonradioactive southwestern analysis using Cemmiluminescent detection, Biotechniques **13**, 540—543 (1992).

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

11) S. Ikeda and T. Oda : Nonisotopic gel-mobility shift assay using chemiluminescent detection system, Biotechniques **14**, 878—880 (1993).

12) J. Sambrook, E.F. Fritsch and T. Maniatis ; Extraction of bacteriophage λ DNA. in Molecular Cloning, A Laboratory Manual, pp2.80-2.81, Cold Spring Harbor Laboratory Press, New York (1989).

13) P. Michael and L. Andrew : PCR base diagnosis in presence of 8% (v/v) blood, Nucleic Acids Res. **19**, 1151 (1991).

14) J.D. Watson, M. Gilman, J. Witkowski and M. Zoller : The polymerase chain reaction. in Recombinant DNA, p.79-98, W.H. Freeman and company, New York (1992).

15) C. Yanisch-Perron, J. Vieira and J. Messing : Improved M13 phage cloning vectors and host strains : Nucleotide sequences of the M13 mp18 and pUC 19 vectors, Gene **33**, 103—119 (1985).

16) S. Ikeda , H. Sumiyoshi and T. Oda : DNA binding properties of recombinant mitochondrial transcription factor 1, Cell. Mol. Biol. **40**, 483—493 (1994).

17) R.A. Young and R.W. Davis : Efficient isolation of genes using antibody probes, Proc. Natl. Acad. Sci. USA **80**, 1194—1198 (1983).

18) S. Anderson, A.T. Bankier, B.G. Barrell, M.H.L de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H Smith, R. Staden and I.G. Young : Sequence and organization of the human mitochondrial genome, Nature **290**, 457—465 (1981).