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Purification and Characterization of Human Mitochondrial Transcription Factor 1

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We purified to near homogeneity a transcription factor from human KB cell mitochondria. This factor, designated mitochondrial transcription factor 1 (mtTF1), is required for the in vitro recognition of both major promoters of human mitochondrial DNA by the homologous mitochondrial RNA polymerase. Furthermore, it has been shown to bind upstream regulatory elements of the two major promoters. After separation from RNA polymerase by phosphocellulose chromatography, mtTF1 was chromatographed on a MonoQ anion-exchange fast-performance liquid chromatography column. Analysis of mtTF1-containing fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single major polypeptide with an M_r of approximately 25,000. Centrifugation in analytical glycerol gradients indicated a sedimentation coefficient of ~2.5 S, consistent with a monomeric 25-kilodalton protein. Finally, when the 25-kilodalton polypeptide was excised from a stained sodium dodecyl sulfate-polyacrylamide gel and allowed to renature, it regained DNA-binding and transcriptional stimulatory activities at both promoters. Although mtTF1 is the only mitochondrial DNA-binding transcription factor to be purified and characterized, its properties, such as a high affinity for random DNA and a weak specificity for one of its target sequences, may typify this class of regulatory proteins.

The problem of transcriptional promoter selection has been a major focus of molecular biology. By studying how cells specify and regulate the accurate initiation of transcription, we can begin to understand how they establish and maintain tissue- and developmental stage-specific patterns of gene expression. Two general approaches are taken toward the isolation and characterization of transcriptional specificity factors. Genetic analysis allows the unambiguous assignment of transcriptional function to a defined chromosomal locus, based on phenotypic effects of perturbing or ablating that locus. Subsequent biochemical isolation of wild-type and mutant gene products can reveal the mechanism of transcriptional stimulation or repression (e.g., DNA binding), but these effects can rarely be fully reconstituted in vitro. In contrast, direct biochemical strategies, which are pursued in systems in which genetic manipulations are impractical or currently impossible, have recapitulated aspects of in vivo promoter recognition and regulation in cell extracts, but have led to the identification and detailed molecular characterization of specific effector proteins in only a few instances.

Studies of promoter selection in human mitochondrial DNA (mtDNA) have been limited to biochemical analyses of promoter sequences and the transcriptional apparatus that recognizes them in soluble organelle extracts (4, 7, 11, 13, 14, 21, 33). At least one specificity factor, in addition to mitochondrial RNA (mtRNA) polymerase, is required for efficient promoter recognition in vitro (13). This mitochondrial transcription factor (mtTF) binds to upstream regulatory elements of both major promoters of human mtDNA, thereby activating them. Sequence comparison of the two elements, of the heavy (H)- and light (L)-strand promoters (HSP and LSP, respectively), has suggested that mtTF could function in an orientation-independent manner (14). However, it remained formally possible that the similarity between the HSP and LSP regions was fortuitous and that the ability of partially purified mtTF to activate both promoters (or, indeed, that its ability to bind DNA and to stimulate transcription) reflected the functions of multiple distinct factors. Here we report the purification of mtTF nearly to homogeneity, through conventional ion-exchange chromatographies, without resolution of distinct promoter-specific functions. The highly purified factor bound specifically to both promoter regions and retained both HSP- and LSP-stimulatory activities. Electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels revealed a single major polypeptide with a molecular mass of approximately 25 kilodaltons (kDa) that, when excised from the gel and allowed to renature, performed all the functions previously ascribed to mtTF. The implications of a common DNA-binding factor for the control of H- and L-strand transcription and, perhaps, H-strand replication are considered.

MATERIALS AND METHODS

Plasmid templates for in vitro transcription and DNA-binding assays. Construction of recombinant plasmids containing either major promoter (or both major promoters) of human mtDNA has been described previously (7, 13, 14). Digestion of these plasmids with restriction endonucleases to yield runoff transcription templates and substrates for end labeling (for DNA binding assays) was also done as reported previously (13, 14). The individual clones used in each assay are indicated in the respective figure legends. Plasmid L5′Δ-70 (7) carries an intact LSP only and was used to generate both the LSP-specific probe for DNA-binding activity and the nonspecific probe, an ~270-base-pair (bp) *Bam*HI-*Sal*I fragment of the pBR322 vector. Plasmid H5′Δ-60 (7) contains an intact HSP only and was used to generate HSP-specific end-labeled DNA fragments. Plasmid pKB741SP (13) contains both the wild-type LSP and HSP and was digested with *Eco*RI to produce a runoff template encoding a 416-nucleotide L-strand transcript and a 190-nucleotide H-strand transcript. The end-labeled fragment containing both the intact LSP and HSP, which was used in the experiment depicted in Fig. 2, was also derived from pKB741SP by digestion with *Ava*II and *Bal*I. Finally, plasmid LS-28/-37

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bears an intact HSP and a mutant LSP harboring a linker substitution in the mtTF1-binding site, as reported previously (14).

In vitro transcription. Transcription of linearized templates containing mtDNA promoters and processing of and visualization of the RNAs that were produced were performed by previously published methods (13, 14).

DNase I footprinting. Sequence-specific binding of mtTF to end-labeled mtDNA fragments containing LSP, HSP, or both was analyzed by a modification of the DNase I protection method of Galas and Schmitz (17), as described previously (14).

Gel electrophoretic analysis of DNA binding. We used the gel system described by Sharp and co-workers (6) to resolve mtTF-DNA complexes. Mitochondrial protein fractions were incubated with 0.5 to 2 ng of various end-labeled restriction fragments (the identities of the fragments and the exact concentrations used are reported for each experiment in the respective figure legends) in a reaction volume of 10 μl, containing 10 mM Tris hydrochloride (pH 8.5), 10 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), and 100 μg of RNase-free bovine serum albumin (BSA) per ml. Reactions were incubated at 28°C for 20 min; 1 μl of 50% glycerol containing bromphenol blue and xylene cyanol tracking dyes was added and gently mixed into the solution; and samples were loaded directly onto 4% acrylamide (0.11% bisacrylamide) slab gels and electrophoresed for 3 h at 30 mA in 6.7 mM Tris hydrochloride (pH 7.9)–3.3 mM sodium acetate–1 mM EDTA. Gels were preelectrophoresed for 2 h at 20 mA, and buffer was recirculated during both prerunning and sample electrophoresis. Gels were dried and autoradiographed.

For DNase I protection analysis of the mtTF-DNA gel complexes, the basic method described above was modified as follows. Preparative DNA-binding reactions were performed in a volume of 80 μl, with all concentrations of reaction components as described above, except that linearized plasmid DNA (vector pSP64) was included at a concentration of 1 μg/ml. After preincubation at 28°C for 20 min, samples were treated with pancreatic DNase I (Worthington Diagnostics, Freehold, N.J.) at a concentration of 2 μg/ml for 30 s at room temperature. Digestion was stopped by the addition of EDTA to a final concentration of 12 mM, the tubes were placed on ice, and their contents were quickly loaded onto a nondenaturing gel and electrophoresed as described above. None of these manipulations (following preincubation) has a significant effect, either qualitative or quantitative, on mtTF-DNA binding, as measured by the distribution of radioactive fragments in free and bound forms in nondenaturing gels (data not shown). The wet gel was autoradiographed, and gel slices corresponding to the various complexes (see Fig. 2) were excised. Labeled DNA was eluted from gel slices by the method of Carthew et al. (6), extracted once with phenol-chloroform and once with chloroform, and precipitated with ethanol. Recovery from each complex was determined by Cerenkov counting of the dried pellets, and the DNA was dissolved in 80% formamide–TBE (45 mM Tris borate [pH 8.3], 1 mM EDTA)–0.01% bromphenol blue–0.01% xylene cyanol, to obtain solutions with equivalent specific activities (equal counts per minute per microliter). Samples were denatured at 70°C for 5 min, and 10 μl of each was electrophoresed in 7 M urea–8% polyacrylamide sequencing gels at 1,000 V in TBE. The gels were transferred to DE81 paper (Whatman, Inc., Clifton, N.J.) dried, and fluorographed. Chemical sequencing reactions (25) were carried out with identical labeled fragments, and cleavage products were electrophoresed alongside DNase digestion products, in order to map gel complex footprints within the mtDNA sequence.

Purification of mtTF from KB cells. (i) S-130 preparation. A soluble extract of human mitochondrial enzymes was prepared essentially as described previously (13). Mitochondria were isolated by the sucrose step gradient method (3), pelleted, frozen in liquid nitrogen, and stored at −70°C. For a typical purification, mitochondria from 40 to 60 liters of KB cells were thawed on ice, washed once with ~300 ml of wash buffer (20 mM Tris hydrochloride [pH 8.0]; 0.2 mM EDTA; 1 mM DTT; 1 mM phenylmethylsulfonyl fluoride; 0.25 M sucrose; and 15% glycerol containing 0.1 μg of each of the following per ml: antipain, chymostatin, elastatinal, leupeptin, and pepstatin), and centrifuged in a JA-20 rotor (Beckman Instruments Inc., Palo Alto, Calif.) for 15 min at 15,000 rpm (18,000 × g_{av}). The pelleted mitochondria were then suspended in 30 ml of lysis buffer (which was the same as the wash buffer described above, except that sucrose was omitted). The volume of the suspension was adjusted to 53 ml with deionized H_2O, and the mitochondria were lysed by the addition of 1.5 ml of 20% (vol/vol) Triton X-100 (final concentration, 0.5%) and homogenized by 10 strokes in a glass homogenizer with a tightly fitting motor-driven Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) pestle. The KCl concentration was raised to 0.35 M by the addition of 5.25 ml of 4 M KCl, and the homogenization was repeated. The lysate was centrifuged for 60 min at 45,000 rpm (130,000 × g_{av}) in a Ti75 rotor (Beckman), and the supernatant, designated S-130, was removed carefully.

(ii) DEAE-Sephacel chromatography. S-130 was diluted 3.5-fold with buffer A (10 mM Tris hydrochloride [pH 8.0]; 0.1 mM EDTA; 1 mM DTT; 7.5% glycerol; 0.1 μg each of antipain, chymostatin, elastatinal, leupeptin, and pepstatin per ml; and 0.5 mM phenylmethylsulfonyl fluoride), to reduce the KCl concentration to ~0.1 M, and loaded onto a 60-ml DEAE-Sephacel column, which was prepared according to the instructions of the manufacturer (Pharmacia, Inc., Piscataway, N.J.), and equilibrated in buffer B (which was the same as buffer A, except that phenylmethylsulfonyl fluoride was omitted)–0.1 M KCl. The column was then washed with buffer B–0.1 M KCl until a constant A_{280} by the effluent was recorded. Next, the column was washed with buffer B–0.3 M KCl, which eluted both mtTF and mtRNA polymerase (data not shown), and ~1-ml fractions were collected and assayed for RNA polymerase activity by using poly(dA-dT) (P-L Biochemicals, Inc., Milwaukee, Wis.) as the template. A pool of active fractions identified by this assay retains full promoter selectivity when assayed with either HSP- or LSP-containing templates (unpublished observations).

(iii) Phosphocellulose chromatography. The DEAE pool was loaded directly onto a 25-ml column of phosphocellulose P11 that was precycled according to the instructions of the manufacturer (Whatman) and equilibrated in buffer B–0.3 M KCl. After extensive washing with 0.3 M KCl to remove unbound material, the column was developed with a linear gradient of KCl concentrations (0.3 to 1.0 M) in 200 ml of buffer B at a flow rate of 10 ml/h, and ~4-ml fractions were collected. Fractions were assayed for both nonspecific [poly(dA-dT)-dependent] and specific (HSP- or LSP-driven, mtTF-dependent) RNA polymerase activities which cochromatographed with ~0.5 M KCl. Fractions were also assayed for the stimulation of promoter-specific transcription catalyzed by factor-depleted RNA polymerase and for DNA binding by both DNase I footprinting and gel retardation.

Binding and stimulatory activities reproducibly fractionated nearly, but not exactly, together at this stage, with the stimulatory activity peaking one fraction ahead of the binding activity with ~0.7 M KCl.

(iv) MonoQ FPLC. Fractions containing mtTF activities (transcription stimulatory and DNA binding) that eluted from phosphocellulose (PC) were pooled and concentrated by ultrafiltration by using microconcentrators (Centricon 10; Amicon Corp., Lexington, Mass.). In a typical run, 12 to 16 ml of PC pool derived from ~24 liters of cells was concentrated 10- to 20-fold by centrifugation for 2 to 2.5 h at 6,000 rpm (2,800 × g_{av}) in a JA-20 rotor. The concentrate (~1 ml) was recovered, and the concentrating chambers were rinsed with buffer C (which was the same as buffer B, except that peptide protease inhibitors were omitted). The concentrate was mixed with the rinse, and the volume of this pool was adjusted to ~8 ml with buffer C, in order to reduce the KCl concentration to below 0.1 M KCl. The PC pool was then loaded onto a MonoQ anion-exchange fast-performance liquid chromatography (FPLC; Pharmacia) column equilibrated in buffer C–0.1 M KCl. The column was washed with 10 ml of buffer C–0.1 M KCl and then developed with a linear gradient of KCl concentrations (0.1 to 0.75 M) in buffer C. Fractions of 0.5 ml each were collected and assayed for transcriptional stimulation at both HSP and LSP, DNase I protection at both HSP and LSP, and gel retardation of LSP-containing DNA fragments. The polypeptide composition of column fractions was assessed by SDS-polyacrylamide gel electrophoresis (PAGE), essentially as described by Laemmli (23), followed by silver staining by the method of Wray et al. (37). Protein concentrations were determined by the method of Schaffner and Weissman (28).

Glycerol gradient sedimentation. Fractions containing mtTF eluted from MonoQ were pooled. To 200 μl of the MonoQ pool (~8% of the total or 150 ng of protein) we added 1.0 μl of a 10-mg/ml solution of diethylpyrocarbonate-treated, autoclaved gelatin as the carrier protein. This sample was then layered onto a 4-ml glycerol gradient (20 to 40% [vol/vol]) in buffer D (10 mM Tris hydrochloride [pH 8.0], 300 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 μg of gelatin per ml). After centrifugation for 18 h at 50,000 rpm (250,000 × g_{av}) in an SW60Ti rotor (Beckman), the gradient was fractionated from the bottom by pumping it through a capillary tube. Fractions (200 μl) were assayed immediately for stimulation of promoter-specific runoff transcription and for DNA binding by electrophoretic mobility shift. A mixture of standard proteins was centrifuged in a parallel gradient, which was fractionated as described above. Fractions of this gradient were subjected to SDS-PAGE, and marker proteins were visualized by Coomassie blue staining, in order to calibrate the gradient and thus determine the sedimentation coefficient of mtTF. Standard proteins and their respective, known sedimentation coefficients were as follows: cytochrome *c*, 2 S; BSA, 4.3 S; aldolase, 7.6 S; and catalase, 11.3 S.

Renaturation of mtTF1 purified by SDS-PAGE. To 250 μl of a MonoQ fraction of mtTF (183 ng of protein) we added 25 μg of β-lactoglobulin (Sigma Chemical Co., St. Louis, Mo.) as the carrier protein, 5 μl of 1 M DTT (final concentration, 20 mM), and 1 μl of 25% SDS (final concentration, 0.1%). We precipitated the protein by adding 1 ml of cold acetone and incubating it at −70°C. Following centrifugation (10 min in an Eppendorf microcentrifuge at 4°C), the pellet was dried, suspended in SDS sample buffer, and heated at 55°C to ensure solubilization. Denaturing electrophoresis, gel fractionation, protein elution, acetone precipitation, denaturation with guanidine hydrochloride, and renaturation were performed essentially as described previously (20), with the following modifications and adaptations. We employed slab gels, rather than tubes, and substituted DTT for β-mercaptoethanol in the sample buffer. The separating gel contained 10% polyacrylamide. After electrophoresis, protein was visualized by staining with Coomassie blue, with brief destaining in 7.5% acetic acid. The gel lane containing

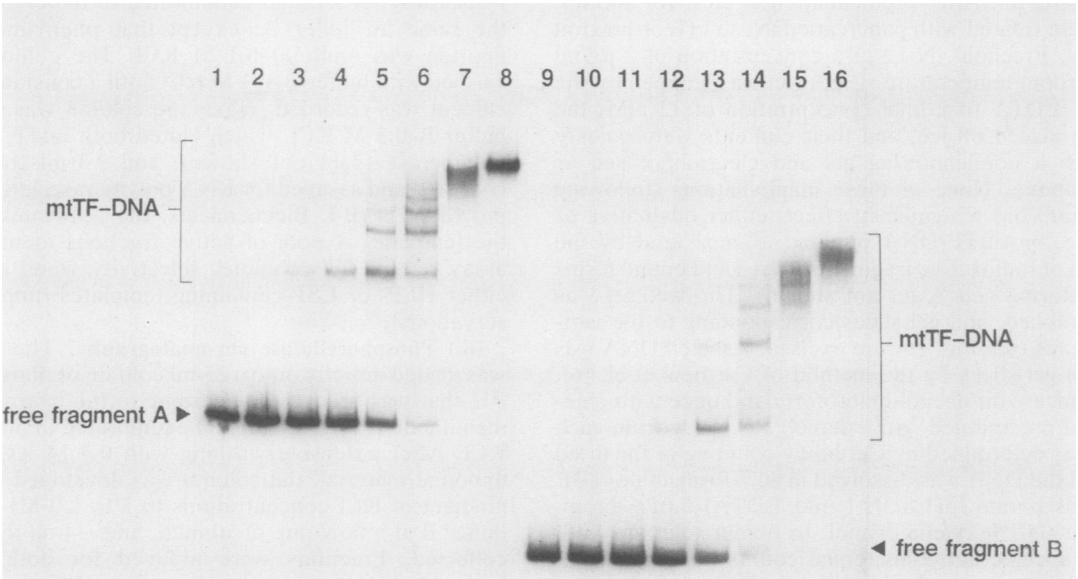


FIG. 1. Detection of mtTF-DNA binding by nondenaturing gel electrophoresis. Two DNA fragments that were 5′ end labeled with ^{32}P were incubated with increasing amounts of PC-purified mtTF and electrophoresed in 4% polyacrylamide gels, as described in the text. Fragment A (lanes 1 to 8) is an ~490-bp *Bam*HI-*Eco*RI fragment containing an intact LSP that was derived from plasmid L5′Δ-70. Fragment B (lanes 9 to 16) is an ~270-bp *Bam*HI-*Sal*I fragment of the pBR322 vector. Factor additions were as follows: none added (lanes 1 and 9), 0.006 μl (lanes 2 and 10), 0.012 μl (lanes 3 and 11), 0.025 μl (lanes 4 and 12), 0.05 μl (lanes 5 and 13), 0.1 μl (lanes 6 and 14), 0.2 μl (lanes 7 and 15), and 0.4 μl (lanes 8 and 16).

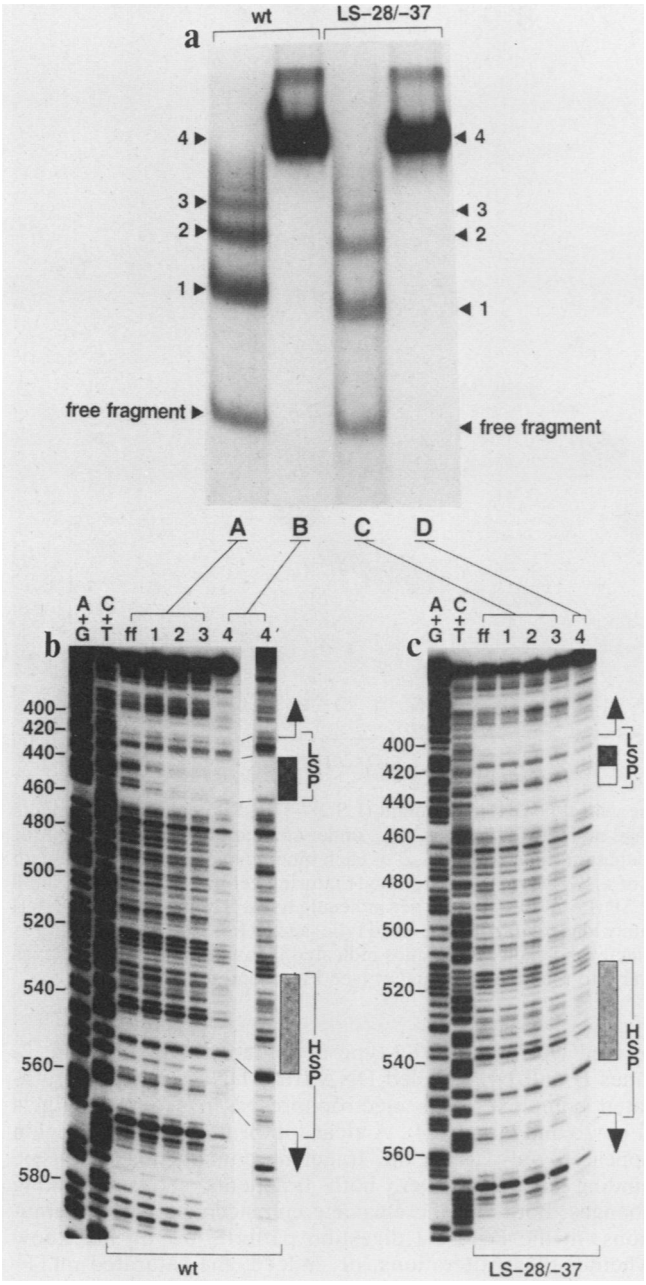


FIG. 2. DNase I protection analysis of mtTF-DNA complexes resolved by gel electrophoresis. Preparative DNA-binding reactions were carried out with 8 ng of an ~330-bp *Ava*II-*Bal*I fragment that was 3′ end labeled with ^{32}P, derived either from pKB741SP (wild type [wt], lanes A and B in panel a and all lanes in panel b) or from linker substitution mutant LS-28/-37 (lanes C and D in panel a and all lanes in panel c). Reactions contained 2 μl (lanes A and C) or 16 μl (lanes B and D) of PC-purified mtTF. After incubation, the DNA-protein mixture was digested with DNase I and electrophoresed as described in the text. The gel was fractionated by using the autoradiogram shown in panel a to locate and excise mtTF-DNA complexes 1 to 4, as well as the uncomplexed free fragment (ff). The DNA was eluted from these gel slices, precipitated with ethanol, denatured, and electrophoresed in the 6% polyacrylamide sequencing gels shown in panels b and c. The lane designations in panels b and c correspond to the number of the complex (or uncomplexed free fragment) from which the DNA was extracted. In lane 4′ in panel b, a twofold excess of radioactivity extracted from complex 4 in lane B, panel a (wild-type fragment), was electrophoresed, to compensate (approximately) for the increased amount of undigested fragment in complex 4. The numbers to the left of panels b and c refer to the published sequences of human mtDNA (1, 32). The hatched vertical bars indicate the mtTF-binding site upstream of the HSP; the solid bars indicate the LSP-proximal mtTF-binding site. In LS-28/-37, the mtTF-binding site was disrupted by substitution of a 10-bp *Bam*HI linker, as indicated by the open portion of the bar on the right in panel c. Arrows mark the position of initiation and direction of transcription from HSP and LSP.

mtTF was cut into 16 5-mm-wide slices approximately spanning the 15- to 150-kDa size range. The gel slices were eluted for ~5 h at 25°C; protein was precipitated with acetone overnight at −70°C; precipitated protein was redissolved in 20 μl of 6 M guanidine hydrochloride in buffer E (10 mM Tris hydrochloride [pH 8.0], 50 mM KCl, 0.1 mM EDTA, 7.5% glycerol, 1 mM DTT, 100 μg of RNase-free BSA per ml). After a 20-min incubation at room temperature, the reactions were diluted 50-fold with buffer E. An additional 20 min at room temperature was allowed for complete renaturation, and fractions were assayed for transcriptional stimulation and DNA binding.

RESULTS

Detection of mtTF activity by electrophoretic retardation of DNA fragments. A major advance in the study of sequence-specific DNA-binding proteins has been the development of the gel retardation assay for the formation of protein-DNA complexes (15). In several instances, this method has been used to identify binding activities in crude extracts by virtue of the pronounced specificities of these factors for their target sequences (6, 10, 19, 26, 27, 31). Therefore, we adapted this technique for detection, and most importantly for quantitation of the mtTF-DNA interaction, as we could not rely for this purpose on transcriptional stimulation at early stages in purification, before the factor was separated from mtRNA polymerase. Figure 1 shows the effect of adding increasing amounts of PC-purified mtTF (see above) on the electrophoretic behavior of two different DNA fragments. As the mtTF concentration was increased, the DNA was recruited to slower-migrating forms. Subsequent experiments showed that this ladder represents mtTF-DNA complexes with different stoichiometries (mtTF:DNA ratios) rather than complexes involving multiple, distinct DNA-binding activities (see Fig. 5 and 7). Remarkably, however, the formation of complexes was not sequence dependent. Qualitatively similar patterns were obtained with labeled fragment A, which contained LSP and which binds mtTF tightly (14), or with fragment B, which contained no mtDNA sequences and no mtTF-binding sites, as defined by protected regions in DNase I footprint analyses (data not shown). The sequence specificity of mtTF-DNA complexes can be demonstrated, however, in two ways. First, as shown in Fig. 2, DNase protection analysis of mtTF-DNA complexes distinguished specific from nonspecific binding. In this experiment, PC-purified mtTF was incubated with a labeled restriction fragment containing either a wild-type LSP or an LSP bearing a *Bam*HI linker substitution in the mtTF-binding site. The mutant sequence of this clone, LS-28/-37, is defective in both footprinting and transcriptional activity (14). At equilibrium, however, the association of mtTF with either LSP (wild type or mutant) resulted in indistinguishable distributions of the DNA in the uncomplexed form and in complexes with increasing stoichiometries (complexes 1 to 3 in lanes A and C, Fig. 2). Before the

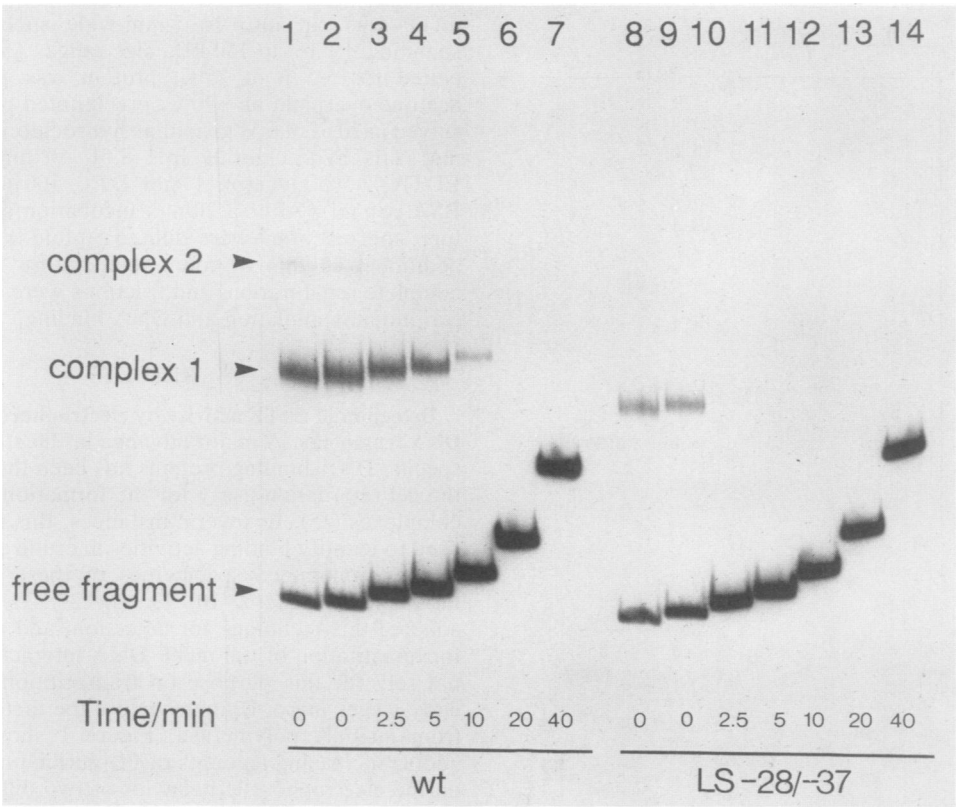


FIG. 3. Stability of specific and nonspecific mtTF-DNA complexes. The wild-type (wt) and mutant (LS-28/-37) LSP-containing fragments (1 ng each; see legend to Fig. 2) were preincubated with 0.1 μl of PC-purified mtTF for 20 min at 28°C, under standard conditions except for the addition of 40 mM KCl, in a total volume of 80 μl. At the end of preincubation (time zero), 10 μl of each binding mixture was mixed with 1 μl of loading dye and loaded onto lanes 1 (wild type) and 8 (LS-28/-37) of a 4% polyacrylamide nondenaturing gel that was running at 30 mA. We then added 700 ng of plasmid DNA containing a single wild-type LSP (L5′Δ-70; see text) per molecule to each of the remaining 70-μl mixtures. Another 10-μl portion was taken from each reaction and immediately loaded onto lanes 2 (wild type) and 9 (LS-28/-37). The mixtures were incubated for 40 min at 28°C; portions (10 μl) were withdrawn and electrophoresed at the times indicated below each lane. Complexes 1 and 2 probably reflect the addition of one and two molecules, respectively, to the labeled fragment (see Fig. 2 and text).

mixture of complexes was loaded onto the nondenaturing gel, it was subjected to limited digestion with DNase I, a treatment that did not affect mobility (data not shown). After electrophoresis and autoradiography of the wet gel, complexes were excised and the DNA was eluted, deproteinized, and electrophoresed in 6% sequencing gels, alongside sequence-specific chemical cleavage products of the same fragments. For both fragments (wild type and mutant) the pattern of DNase I cleavage seen with uncomplexed DNA (which migrated as a free fragment in nondenaturing gels) was identical to that generated from the same fragment in the absence of mtTF (data not shown). However, the retarded complexes formed by mtTF with the wild-type fragment, but not with the mutant, were protected from DNase I cleavage at the previously identified mtTF-binding site of LSP. Significant protection was seen in the first (fastest-migrating) complex (complex 1) and was virtually complete with the next increment in mtTF:DNA stoichiometry (complex 2). However, no discrete protection of the mutant sequence was seen at any mtTF:DNA ratio, even though complexes with similar stoichiometries (similar mobilities in nondenaturing electrophoresis) were readily separated from uncomplexed DNA.

At very high mtTF:DNA ratios, the labeled fragment was quantitatively shifted to a slow-migrating form which could not be further retarded by increased mtTF addition (data not shown). Such a complex (designated here as complex 4) formed with either wild-type or mutant fragments (Fig. 2, lanes B and D). Labeled DNA from DNase I-treated saturated complexes was electrophoresed in sequencing gels (Fig. 2b and c, lanes 4). A clear footprint of the LSP region appeared only when the fragment contained a wild-type binding site. However, both fragments showed striking changes, both specific cleavage enhancements and diminutions, in the DNase I digestion pattern. We do not know whether these alterations, or, indeed, the saturated mtTF-DNA complex itself, have any functional significance. It is interesting that consistent changes in DNase I cleavage occurred in the previously identified mtTF-binding site of HSP. Although weak footprints of this site have been seen in one-dimensional DNase I protection analyses (14), they were not detectable in the unsaturated mtTF-DNA complexes that were analyzed, even when the competing LSP was disrupted (Fig. 2c, lanes ff to 3). At saturation, however, cleavage at specific bases at both the upstream and downstream boundaries of the HSP-proximal mtTF response element was markedly enhanced, with perhaps some discrete protection of the site itself (Fig. 2, lanes 4 and 4′; the vertical hatched bar delimits the putative protected region of the HSP; the vertical solid bar delimits the sequences protected at LSP).

Specific mtTF-LSP complexes were also distinguished from nonspecific mtTF-DNA complexes by virtue of their enhanced stabilities to dissociation in the presence of unla-

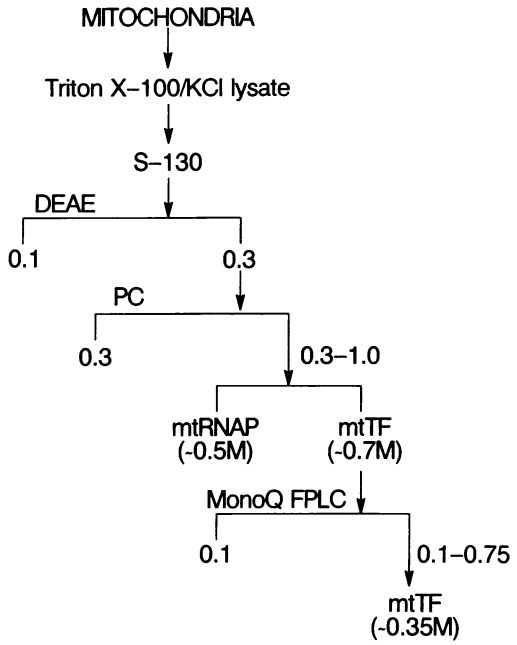


FIG. 4. Purification of mtTF from human mitochondria. An extract of human mitochondria was chromatographed on three successive ion-exchange resins, as described in the text. In the first chromatography (DEAE-Sephacel), mtTF was eluted with an ionic strength step. The next two columns (PC and MonoQ FPLC) were developed with KCl concentration gradients, as indicated, and mtTF activity was recovered in eluates at the indicated ionic strengths. Mitochondrial RNA polymerase (mtRNAP) eluted from PC well before mtTF did, as shown.

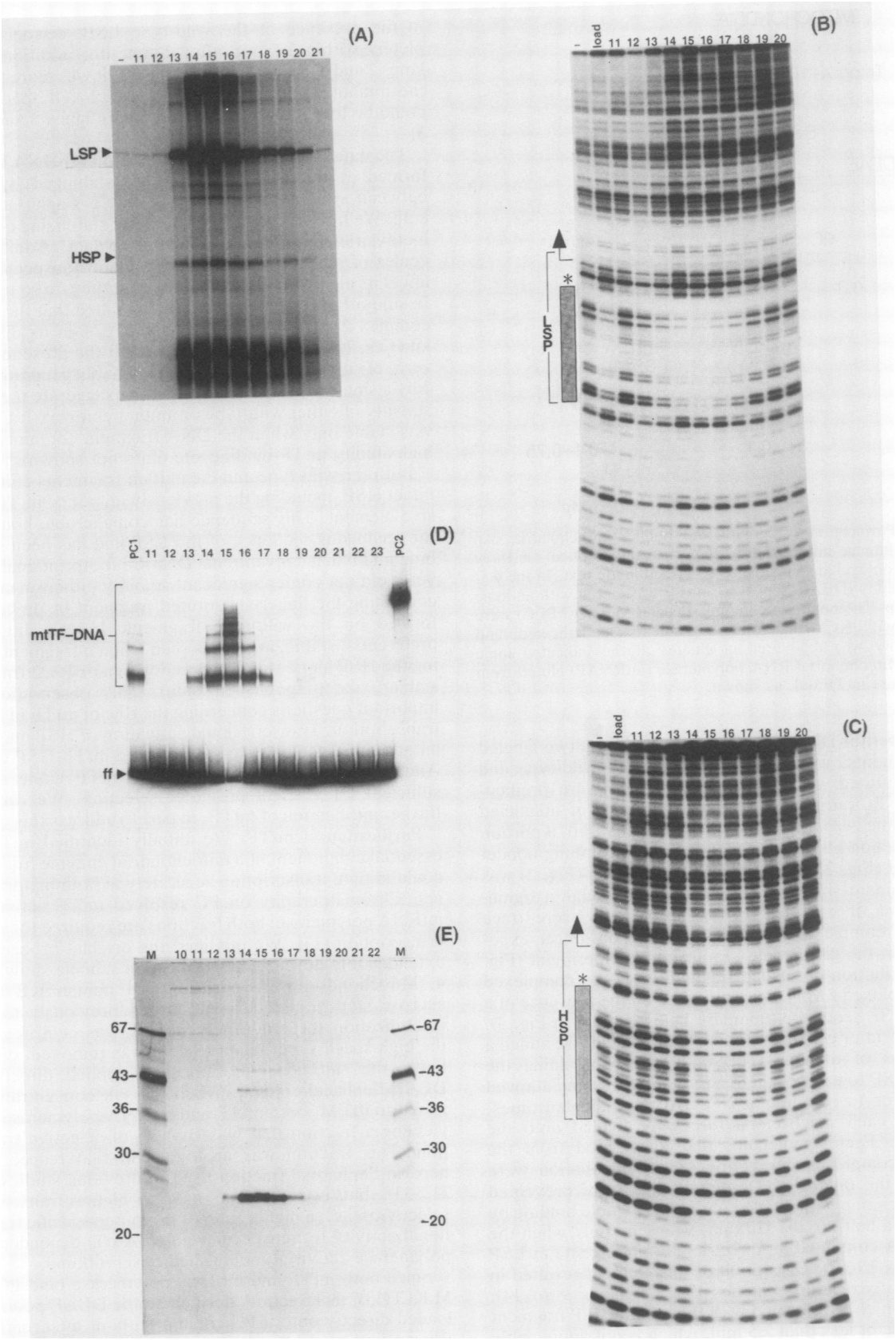
beled competitor DNA (Fig. 3). Here, the same two labeled DNA fragments, which were derived from wild-type and linker-substituted mtDNA clones (Fig. 2), were preincubated with PC-purified mtTF in the absence of unlabeled DNA. At time zero (the end of the preincubation), a portion of each reaction mixture was loaded onto a running nondenaturing gel (Fig. 3, lanes 1 and 8). Complex 1 (Fig. 2) was the predominant shifted species formed, although a significant amount of complex 2 was present, as were trace amounts of complex 3 (which was visible on longer exposures). As in the gel complex footprinting experiment (Fig. 2), the distribution of labeled fragment between complexed and uncomplexed forms did not depend on the presence of a wild-type LSP sequence (compare lanes 1 and 8 in Fig. 3). Immediately after gel lanes 1 and 8 were loaded, we added a large excess of unlabeled plasmid DNA containing the wild-type LSP to each of the reaction mixtures and allowed the incubation to continue at 28°C. At the indicated times, portions of each reaction mixture were loaded onto the running gel. Factor molecules that dissociated from the preformed complexes during the second incubation were trapped by the unlabeled DNA and thus were prevented from reassociating with labeled fragments. Stable binding by mtTF, with a half-life of ~10 min, was seen only when the labeled DNA contained an intact mtTF-binding site at LSP (Fig. 3, lanes 2 to 7). Disruption of this element resulted in accelerated dissociation kinetics (Fig. 3, lanes 9 to 14); complex 1 which was formed with the mutant LSP fragment had a half-life of less than 2.5 min, which is not appreciably longer than that with the fragment of vector DNA (B in Fig. 1) used to detect and characterize nonspecific binding (data not shown). In contrast, complex 2 was quite unstable even in the presence of the wild-type LSP, dissociating completely within 2.5 min after competitor addition (compare lanes 2 and 3 in Fig. 3). Nevertheless, it was more stable than the mutant complex 2, much of which fell apart before it could be loaded onto the gel (compare lanes 8 and 9 in Fig. 3).

The data in Fig. 3 suggest that the mtTF-DNA complexes formed at the wild-type LSP are stabilized by contacts between specific bases and probably a single factor molecule. We have recently mapped these stabilizing contacts by methylation interference (unpublished data). Additional molecules of mtTF can bind this fragment nonspecifically and affect its electrophoretic mobility, but they do so transiently. In support of this interpretation, we noted that the wild-type complex 2 resembled mutant complex 1 in its dissociation kinetics. It should be pointed out that the presence, at time zero, of complex 2, which can presumably generate complex 1 by a single dissociation event, can exaggerate the apparent stability of either wild-type or mutant complex 1. Complex 2 was stable only when the DNA fragment contained another high-affinity mtTF-binding site (data not shown).

Both the wild-type and the mutant fragments contained an intact HSP, including the previously identified mtTF-responsive element. This sequence has considerable homology to the analogous sequence of the LSP and has been shown to bind mtTF in a conventional DNase I footprint analysis (14), yet it did not confer significant stability either on complex 1 containing the linker-substituted fragment or on complex 2 formed on the wild-type fragment. Indeed, little or no preferential stabilization was seen when a fragment containing the HSP alone was compared with a pBR322 fragment of similar size (unpublished data). This observation raises important questions concerning the role of mtTF in H-strand transcription. Transcriptional activation of HSP may require an additional factor(s) to stabilize the mtTF-HSP complex. Alternatively, transient binding by mtTF alone could be sufficient for efficient promoter selection. We carried out further purification of mtTF, in part to address this question.

Purification of mtTF. We initially identified mtTF as an essential component of a mitochondrial enzyme fraction that could initiate transcription accurately at both major promoters. Chromatography on PC resolved mtTF activity from mtRNA polymerase; both fractions are required for selective transcription (13). We modified the original procedure and extended it to develop the purification scheme shown in Fig. 4. Mitochondria were purified from human KB cells on sucrose step gradients as described previously (3). After solubilization the crude homogenate was centrifuged; and the high-speed supernatant (S-130) was removed, diluted to lower the salt concentration, and applied directly to a DEAE-Sephacel column. When the salt concentration was raised to 0.3 M, both mtTF and mtRNA polymerase eluted, yielding a fraction that was capable of selective transcription. This fraction was analogous in that respect to the heparin-Sepharose fraction used in previous studies (7, 13, 21, 33), but it seemed to have a higher transcriptional selectivity, i.e., a higher ratio of promoter-specific transcriptional activity to nonspecific, poly(dA-dT)-dependent RNA polymerase activity (unpublished observations).

Since both mtTF and mtRNA polymerase bind PC at 0.3 M KCl (13), this fraction, designated the DEAE pool, can be loaded directly onto a PC column without intervening dialysis or dilution. Elution with a linear salt gradient separated mtRNA polymerase from mtTF. The PC-purified factor stimulated transcription from both HSP and LSP and has been shown to bind domains of both promoters in DNase I


(A)
- 11 12 13 14 15 16 17 18 19 20 21
LSP
HSP
(B)
- load 11 12 13 14 15 16 17 18 19 20
HSP
(C)
- load 11 12 13 14 15 16 17 18 19 20
HSP
(D)
PC1 11 12 13 14 15 16 17 18 19 20 21 22 23 PC2
mtTF-DNA
ff
(E)
M 10 11 12 13 14 15 16 17 18 19 20 21 22 M
67
43
36
30
20
67
43
36
30
20

protection assays (14). The mtTF-containing fractions were pooled, concentrated by ultrafiltration, and applied to a MonoQ anion-exchange FPLC column. Proteins were separated by elution with a linear salt gradient, and fractions were assayed for activities that were present in the PC-purified mtTF fraction (Fig. 5). The fluorogram in Fig. 5A shows the products of runoff transcription synthesized by mtTF-depleted mtRNA polymerase supplemented with MonoQ FPLC fractions. The template that was used contained both a wild-type HSP and a wild-type LSP and yielded runoff products of 190 and 416 nucleotides, respectively. As in the previous step (PC), HSP- and LSP-stimulatory activities cochromatographed exactly. Moreover, the peak of stimulatory activity was coincident with maximal DNA-binding activity, which was assessed by DNase I protection at both LSP (Fig. 5B) and HSP (Fig. 5C). HSP binding was most clearly indicated by the strong enhancement of cleavage (indicated by an asterisk) at nucleotide 547 ± 1 (Fig. 5C), which was 12 bp upstream of the transcriptional start site. Similar cleavage enhancement was seen at LSP (asterisk, Fig. 5B), but was accompanied by much stronger protection of the upstream region. That DNA-binding and transcriptional stimulation are mediated by the same activity is strongly suggested by comparison of the chromatographic profile of stimulation (Fig. 5A) with the mobility shift profile presented in Fig. 5D. Not only did the major peaks of stimulatory and binding activities coincide in fraction 15 (~0.35 M KCl) but a second minor peak of transcriptional activity in fraction 19 was also accompanied by a minor peak of binding activity that was too small to be detected by the less-sensitive footprint analysis. Interestingly, the ladder of bands seen with PC-purified mtTF (Fig. 1 and 2) was also formed with the more highly purified preparation and, in fact, provided a visual indicator of the mtTF concentration in successive fractions. This supports the assertion that different stoichiometries of mtTF to DNA are solely responsible for all the complexes that were formed. Finally, we noted that the symmetry of the elution profile of DNA-binding activity corresponded to the symmetric profile of transcriptional stimulation.

The polypeptide composition of the MonoQ-purified mtTF was analyzed by SDS-PAGE; a silver-stained gel is shown in Fig. 5E. A single major polypeptide with a molecular mass of approximately 25 kDa copurified with the mtTF activity. Some larger species were also present in the mtTF-containing fractions, but they did not correlate with activity. These polypeptides, furthermore, did not appear to be abundant enough to account for the DNA-binding activity of these fractions, although differential silver-staining could have caused underestimation of their abundances.

MonoQ fractions containing mtTF were pooled and sedimented through glycerol gradients (Fig. 6). A mixture of standard proteins was centrifuged in a parallel gradient to allow size determination. Again, HSP- and LSP-specific transcriptional activities (Fig. 6A) and DNA-binding activity (Fig. 6B) all copurified, sedimenting slightly faster than cytochrome *c* (2 S) and well behind BSA (4.3 S). The estimated sedimentation coefficient of 2.5 S (Fig. 6C) was consistent with that of a monomeric 25-kDa protein. However, even when sedimentation analysis was performed in the presence of gelatin as the carrier, substantial losses of activity and protein resulted, precluding the detection of the 25-kDa polypeptide by SDS-PAGE (data not shown).

A single polypeptide has DNA-binding and transcriptional stimulatory activities. In order to identify the polypeptide(s) responsible for mtTF activity, we employed the technique of Hager and Burgess (20) for renaturation of proteins after SDS-PAGE. MonoQ-purified mtTF was denatured in SDS and electrophoresed in 10% polyacrylamide gels. Proteins were visualized by Coomassie blue staining (Fig. 7A). Lane 2 of the gel in Fig. 7A contained 25 μg of a commercial preparation of β-lactoglobulin, which was used here as the carrier protein; the additional bands detectable in Fig. 7A, lane 1, were due to the presence of mtTF in lane 1 only. As noted above, a major species migrated at ~25 kDa, while an additional cluster of minor species was seen at ~100 to ~150 kDa. A duplicate of lane 1 of the wet gel was fractionated into 5-mm-wide slices, as indicated (we deliberately included all of the 25-kDa band in one fraction, fraction 3), and protein was recovered as described above. The fractions were then assayed for their ability to direct selective transcription of both HSP and LSP from a linear template containing both promoters (Fig. 7B) and for DNA-binding activity assayed by DNA fragment retardation in nondenaturing electrophoretic gels (Fig. 7C). In both cases, activity was recovered in fraction 3, which corresponds to the major polypeptide component of MonoQ-purified mtTF. The gel-purified mtTF was active at both promoters (Fig. 7B) and formed the multiple discrete complexes seen throughout purification (Fig. 7C; data not shown: all complexes formed by MonoQ-purified mtTF in Fig. 5D were also generated with the renatured 25-kDa protein). We estimated the recov-

FIG. 5. MonoQ FPLC of mtTF. Fractions eluted from MonoQ with a linear KCl gradient were assayed for stimulation of runoff transcription catalyzed by mtTF-depleted mtRNA polymerase from both the LSP and HSP (A); for DNA binding, by footprint analysis using labeled LSP-containing (B) and HSP-containing (C) fragments and by electrophoretic retardation of an LSP-containing fragment (D); and for polypeptide composition by SDS-PAGE followed by silver staining (E). (A) Runoff transcription was catalyzed by 2 μl of PC-purified (mtTF-depleted) mtRNA polymerase in a total volume of 25 μl with no other fraction added (−) or with 1 μl of MonoQ FPLC fractions 11 to 21 added, as indicated above each lane. The template was *Eco*RI-digested pKB741SP at 1 μg/ml that contained both a wild-type LSP and a wild-type HSP. The expected mobilities of full-length runoff transcripts from both promoters are indicated by arrowheads. Also visible are multiple additional bands representing paused, terminated, or processed transcripts. (B) MonoQ FPLC fractions were assayed for footprinting at LSP. Reactions contained a 5′-end-labeled *Bam*HI-*Eco*RI fragment bearing the wild-type LSP derived from plasmid L5′Δ-70 at 0.1 μg/ml (total DNA concentration, 1.1 μg/ml) in a volume of 20 μl. In the leftmost lane (−), the fragment was digested in the absence of mitochondrial proteins; in the next lane (load), the fragment was digested in the presence of 2 μl of PC-purified mtTF. The remaining lanes contained 2 μl of the MonoQ chromatographic fraction indicated above each lane. The vertical hatched bar delineates mtTF-binding site of LSP, while the asterisk denotes mtTF-induced DNase I cleavage enhancement. The arrow marks the transcriptional start site, and its direction indicates the direction of L-strand transcription. (C) Same as described above for panel B, except that labeled DNA was a *Bam*HI-*Eco*RI fragment of plasmid H5′Δ-60 containing a wild-type HSP and was present at 0.05 μg/ml (total DNA concentration, 0.3 μg/ml). Furthermore, 4 μl of a protein fraction (either PC-purified mtTF or MonoQ FPLC fractions) was added to appropriate reactions. (D) MonoQ FPLC fractions (0.1 μl each) were assayed for binding to an LSP-containing fragment (same as in panel B) by electrophoretic mobility shift. Each reaction contained ~1 ng of labeled fragment in a 10-μl total volume. To provide migration standards, the fragment was incubated with 0.02 μl (lane PC1) or 0.2 μl (lane PC2) of PC-purified mtTF. ff, Free fragment. (E) MonoQ FPLC fractions 10 to 22 (100 μl each) were denatured in SDS, alkylated, and electrophoresed in a 12% polyacrylamide gel. Proteins were visualized by staining with silver. The numbers beside panel E indicate the molecular masses (in kilodaltons) of standard proteins electrophoresed in marker lanes (M).

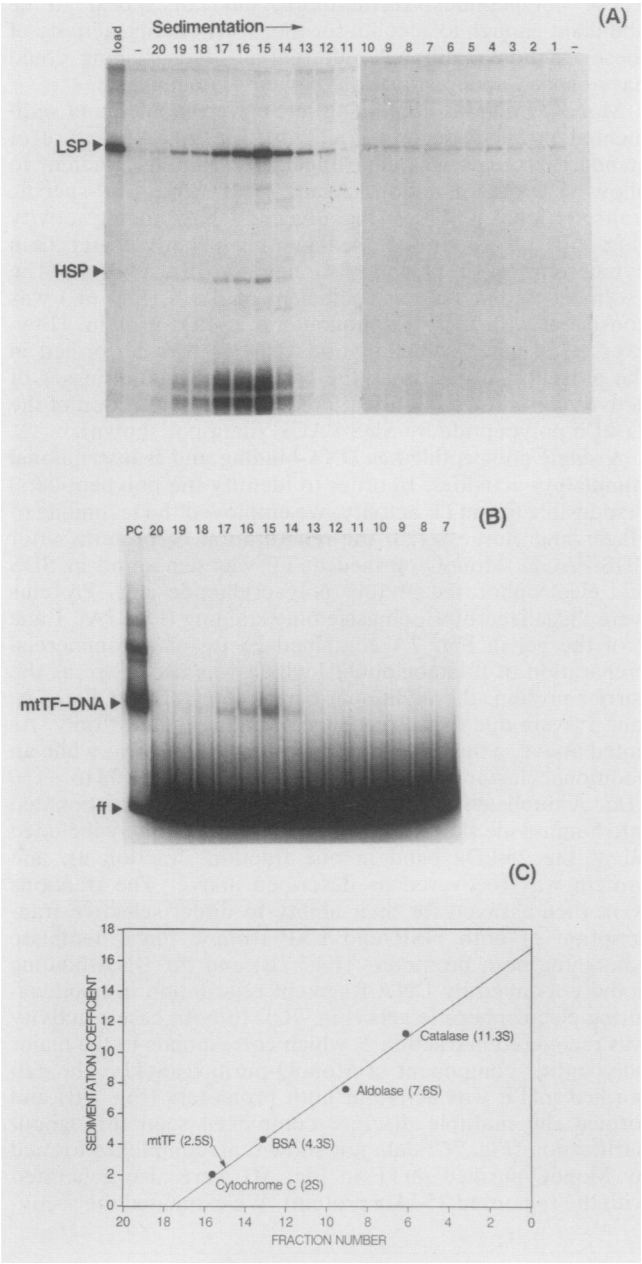


FIG. 6. Glycerol gradient sedimentation of mtTF. MonoQ FPLC fractions containing mtTF activity (Fig. 5) were pooled and sedimented in 20 to 40% glycerol gradients, as described in the text. (A) Gradient fractions were assayed for their ability to stimulate LSP- and HSP-driven transcription catalyzed by PC-purified mtRNA polymerase, as in Fig. 5A. The template was *Eco*RI-digested pKB741SP (4 μg/ml), and the reactions contained 4 μl of MonoQ-purified mtTF (load), a compensating buffer (−), or gradient fractions 1 to 20, as indicated above each lane. (B) Gradient fractions were also assayed for DNA-binding activity by retardation of an LSP-containing labeled fragment in a nondenaturing gel. Individual fractions (7 to 20, as indicated above each lane) were incubated with ~1 ng of an ~160-bp *Bam*HI-*Bal*I fragment of plasmid L5′Δ-70 that was 5′ end labeled with ^{32}P in a reaction volume of 10 μl. As a standard for migration of mtTF-DNA complexes, the same fragment was incubated with 0.05 μl of PC-purified mtTF (lane PC). (C) The sedimentation coefficient of mtTF was estimated, based on the extent of sedimentation of four standard proteins with known sedimentation coefficients in a parallel gradient.

ery of mtTF activity from the gel slice at approximately 50% by titrating a labeled DNA fragment with the active fractions both before and after electrophoretic analysis (unpublished data). No other gel fraction showed significant stimulatory or DNA-binding activity (Fig. 7B and C). Since neither acetone precipitation nor guanidine hydrochloride denaturation or renaturation resulted in any loss of DNA-binding activity (data not shown), the apparent loss in activity was probably due either to irreversible inactivation during electrophoresis, staining, or elution or to incomplete elution of protein from the gel slices.

Regulation of mtDNA promoter selection by mtTF1 in vitro. Efficient transcription from both LSP and HSP thus requires a common factor, which we designated mtTF1. Template commitment assays and deletion analyses, moreover, have established that this factor activates both promoters through sequence-specific DNA binding (14). However, while they share a common domain organization, common effector molecules, and common mechanisms of activation, LSP and HSP differ markedly in their in vitro strength and in their in vivo function (see below). We examined the differential response to mtTF1 of the two promoters present, as they were in vivo, on the same DNA molecule (Fig. 8). The runoff transcription products synthesized by PC-purified mtRNA polymerase in the presence of increasing amounts of MonoQ-purified mtTF1 are shown in Fig. 8A. The specificity and extent of mtTF1-DNA binding were assessed by DNase I protection and gel retardation (Fig. 8B and C, respectively) under conditions that were identical (except for the omission of a labeled nucleoside triphosphate and of mtRNA polymerase) to the transcription conditions (Fig. 8A). At low mtTF1 concentrations, only the LSP was active, producing both a full-length runoff transcript of 86 nucleotides and numerous shorter, presumably paused or processed transcripts. Maximal transcription from the LSP occurred in the presence of 0.5 μl of MonoQ-purified mtTF1 (Fig. 8A, lane 5); at this factor concentration, only a fraction of the mtTF1-binding sites of the LSP were occupied, as determined by DNase I footprinting (Fig. 8B, lane 4). The predominant shifted species was complex 1, but complexes 2 and 3 were also formed (Fig. 8C, lane 5). We note that maximal LSP transcription correlated with maximal complex 1 formation; higher concentrations of mtTF1 recruited the DNA to slower-migrating forms and reduced the amount of full-length L-strand transcript, perhaps by engaging mtRNA polymerase in nonproductive complexes or by inducing the polymerase to pause at internal sites on the template. (Caution should be exercised, however, in interpreting these correlations, as mtRNA polymerase was in limiting concentrations in these reactions.)

HSP of mtDNA behaved very differently in these in vitro mtTF1 titrations. Significant amounts of the full-length HSP-driven runoff transcript (98 nucleotides) were seen only after LSP-dependent transcription reached maximal levels, at or above 0.5 μl of added mtTF1. Maximal HSP-driven transcription was seen with addition of 2 to 3 μl of mtTF1 (Fig. 8A, lanes 7 and 8). This correlated well with the appearance of DNase I cleavage enhancements at both boundaries of the mtTF1-responsive element of HSP (Fig. 8B, lanes 6 and 7; positions of enhancements are denoted by asterisks), supporting the argument that these enhanced cleavages reflect the functional mtTF1-HSP interaction. However, these bonds remained hypersensitive when the amount of mtTF1 added was raised to 4 μl (Fig. 8B, lane 8), even though HSP-driven transcription fell off sharply (Fig. 8A, lane 9). Inhibition of transcription in this case could have been due to

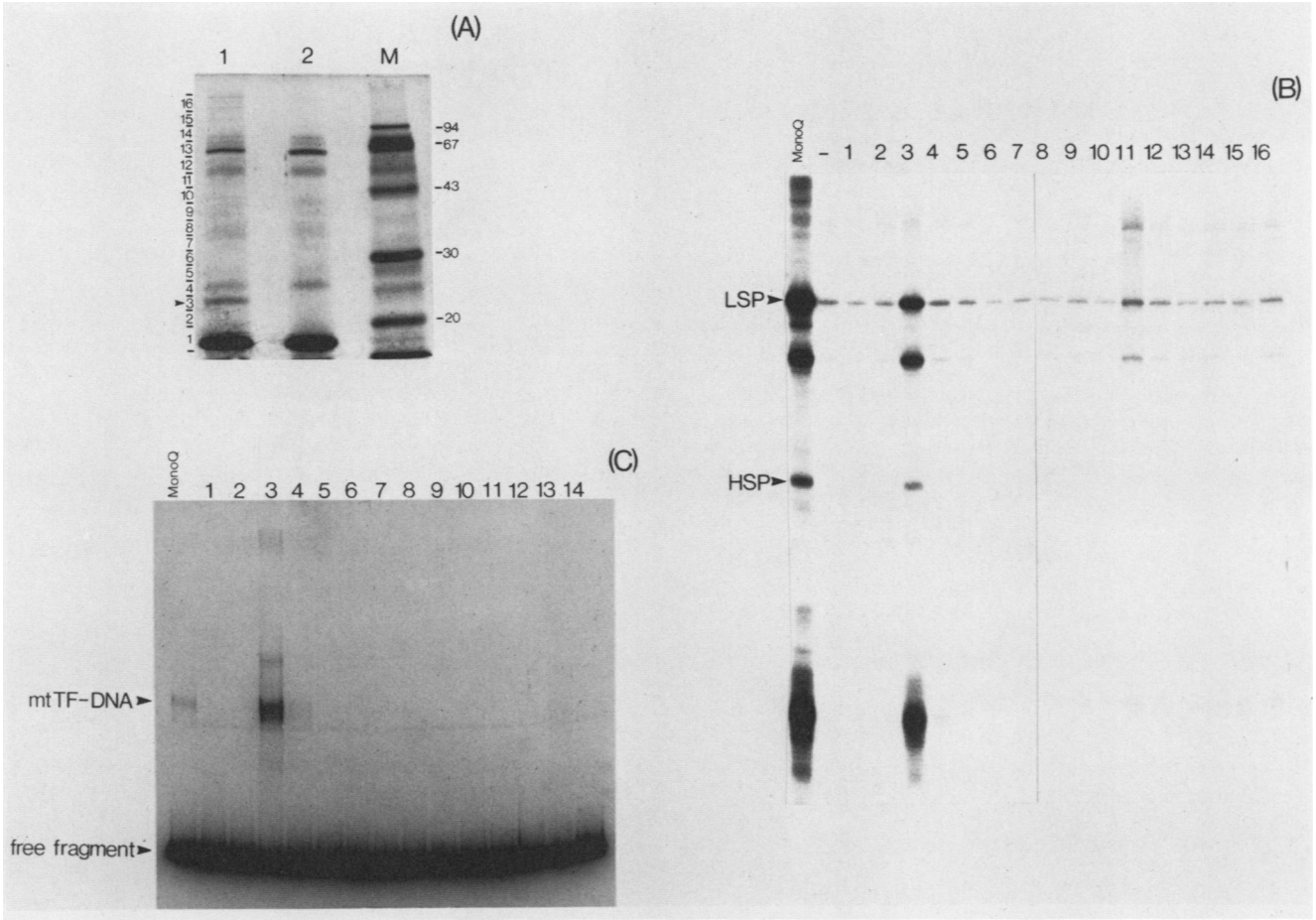


FIG. 7. Renaturation of mtTF1 purified by SDS-PAGE. (A) A pool of MonoQ FPLC fractions containing mtTF activity (250 μl; 183 ng of protein) was denatured in SDS, precipitated with acetone in the presence of carrier protein (25 μg of β-lactoglobulin), redissolved, and electrophoresed in a denaturing 10% polyacrylamide gel, as described in the text. The polypeptide composition of this fraction (plus carrier protein) was visualized by staining with Coomassie blue (lane 1). Lane 2 shows the staining pattern of 25 μg of the carrier protein alone. A duplicate of lane 1 of the wet gel was fractionated, after brief destaining, as indicated on the left. Protein was extracted from each gel slice, precipitated with acetone, denatured with 6 M guanidine hydrochloride, and renatured by dilution, as described in the text. M, Marker. (B) SDS-polyacrylamide gel fractions (4 μl each) were assayed for their ability to stimulate LSP- and HSP-driven runoff transcription catalyzed by 1 μl of PC-purified mtRNA polymerase. The template was *Eco*RI-digested pKB741SP at a concentration of 1 μg/ml. The transcripts synthesized by mtTF-depleted polymerase alone were electrophoresed in the lane indicated by −; stimulation by an equivalent volume (0.73 ng of protein) of the MonoQ-purified mtTF preparation that was not denatured (leftmost lane) allowed a rough estimate of recovery. (C) SDS-polyacrylamide gel fractions (1 μl each) were assayed for binding to an LSP-containing fragment, as in Fig. 5D. The leftmost lane (MonoQ) shows the same fragment reacted with 0.1-volume equivalents of the MonoQ-purified mtTF, while in the rightmost lane (lane 14), 1 μl of a pool of SDS-polyacrylamide gel fractions 14 to 16 was assayed for DNA-binding activity.

nonspecific binding of mtTF1 to the DNA, since gel retardation analysis demonstrated that virtually all of the fragment was complexed with multiple mtTF1 molecules (Fig. 8C, lane 9). At higher ratios of MonoQ-purified mtTF1 to DNA, we saw the global diminution of DNase I cleavage and isolated, specific enhancements characteristic of saturated complexes formed by partially purified factor (Fig. 2); in parallel experiments transcription was greatly reduced (data not shown). This distribution of mtTF1 may impede transcription directly or through competition by nonspecific mtTF1-DNA complexes for limiting mtRNA polymerase. It seems likely that maximal HSP activation requires multiple factor binding, as the predominant species present were shifted to very slow migrating forms (Fig. 8C, lanes 7 and 8); it is not clear, however, whether this reflects simple competition between nonspecific and weakly specific (e.g., the HSP mtTF1-responsive element) binding sites for available mtTF1 or an actual requirement for multivalent binding not needed for LSP activation. In either case, the in vitro results suggest that the mtTF1 concentration could regulate mtDNA promoter selection and thereby a switch between transcription and DNA replication in vivo (see below).

DISCUSSION

The identification and purification, essentially to homogeneity, of a transcriptional specificity factor from human mitochondria is a critical first step toward understanding the molecular basis of transcriptional control in this system, in which genetic tools are as yet unavailable. We have heretofore referred to the partially purified activity required for selective transcription in vitro as mtTF to distinguish it from the other essential component: the chromatographic fraction containing mtRNA polymerase (13). With the demonstration that a single polypeptide, with an apparent molecular mass of approximately 25 kDa, can perform all functions ascribed

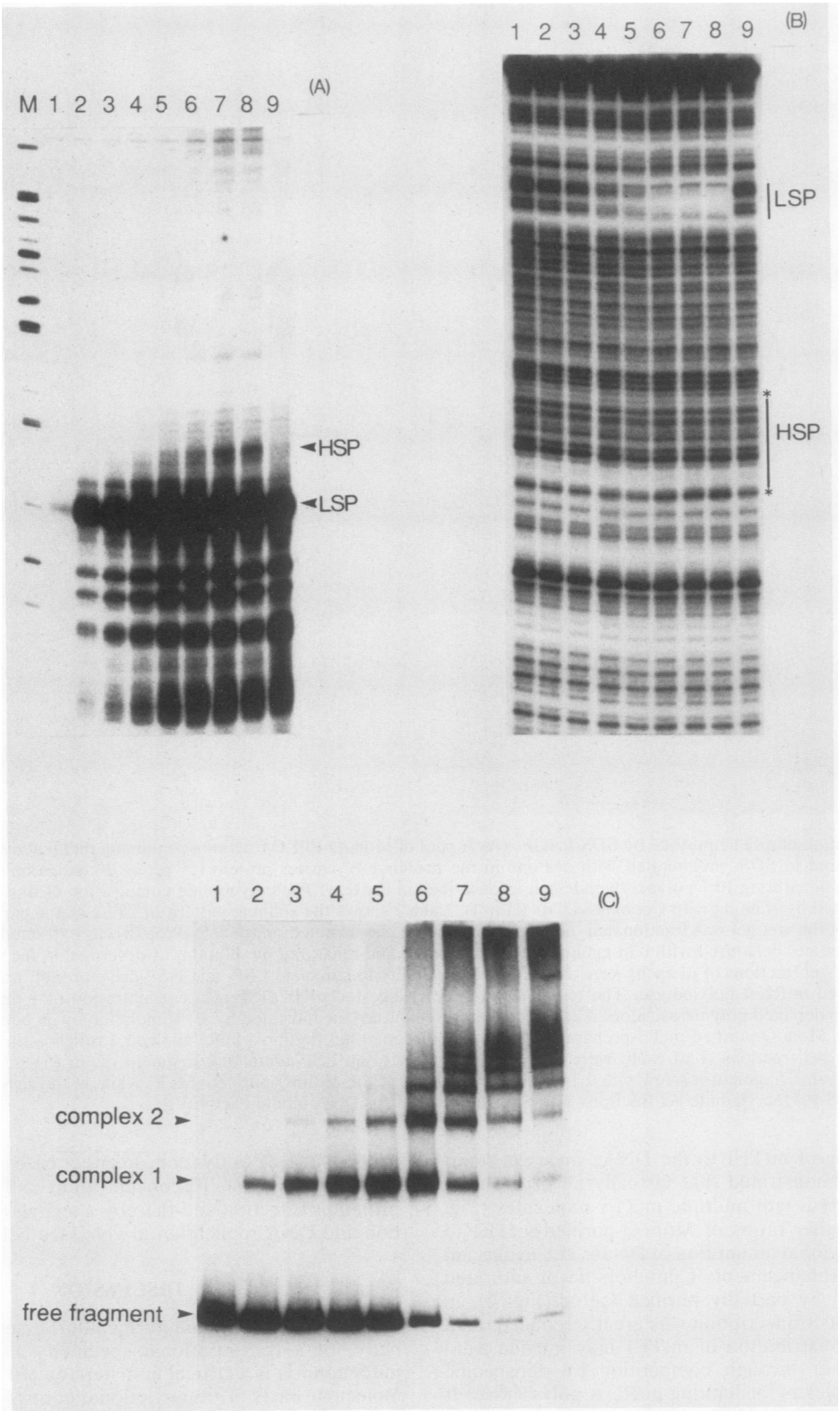


FIG. 8. Regulation of mtDNA promoter selection by mtTF1 in vitro. MonoQ-purified mtTF1 was added in increasing amounts to runoff transcription reactions (A), DNase I protection assays (B), and gel retardation DNA-binding assays (C). In each case the wild-type LSP and HSP were present on the *Ava*II-*Bal*I fragment of plasmid pKB741SP (see text). The fragment used in panels B and C was labeled at the *Ava*II site by Klenow fragment-catalyzed incorporation of [^{32}P]dCMP; the fragment used to program runoff transcription in panel A was incubated with the Klenow fragment and unlabeled deoxynucleoside triphosphates. Both fragments were subsequently isolated on the same agarose gel and included in the mtTF1 assays at equivalent concentrations of ~0.2 μg/ml (theoretical concentration based on 100% yield). Nonspecific carrier DNA (linearized plasmid pSP64) was present at 0.5 μg/ml in all reactions. All reactions (transcriptions, footprinting, and gel

to the partially purified preparation, we will hereafter call this protein mtTF1.

In applying the gel retardation technique to the mtTF1-DNA interaction, we sought a rapid, quantitative assay of factor activity that was independent of transcription. The extreme sensitivity of the assay, while helpful in the detection of small amounts of activity, led to some difficulties in establishing sequence specificity. Unlike DNase I protection analysis, the gel retardation assay allows detection of transient, rapidly dissociating complexes between mtTF1 and nonpromoter DNA, which have the same mobilities as do stable complexes between mtTF1 and fragments bearing a wild-type LSP (Fig. 2 and 3). The absence of any discernible footprints in DNA extracted from these nonspecific complexes indicates that mtTF1 is distributed randomly along the length of the fragment.

We will present a quantitative description of the interactions of mtTF1 with DNA, both specific and nonspecific, in a subsequent report. Here we consider some qualitative observations that were made in the course of purifying mtTF1 and establishing its role in promoter selection. Most striking was the relatively high affinity with which this factor bound random DNA sequences. Nonproductive binding to promoterless DNA was demonstrated by or inferred from several independent assays. Preincubation with pBR322 DNA sequestered the factor, making it unavailable for transcription of a subsequently added promoter template. Similarly, the addition of competitor plasmid DNA to footprinting assays inhibited protection of a labeled LSP at about the same concentration, regardless of whether the competitor contained a promoter (unpublished data). Finally, the affinity for random DNA was shown directly by the gel retardation method, in which both mutant promoters (Fig. 2) and fragments of vector DNA (Fig. 1) bound mtTF1. Is this apparently nonspecific DNA-binding activity inconsistent with the proposed role of mtTF1 in promoter selection? Can it be reconciled with the ability of mtTF1 to recognize a specific DNA sequence and bind it stably, resulting in a DNase I footprint?

In fact, the ability of sequence-specific DNA-binding proteins to associate with random DNA sequences has long been recognized (12, 34). Nonspecific binding is thought to be critical to their function, both by regulating the concentration of free repressors and activators and by enabling them to locate small target sequences within a large genome, essentially by one-dimensional diffusion (16, 22, 36). The unusually high affinity of mtTF1 for random DNA is actually not surprising, considering its probable in vivo function and biosynthetic pathway. Following its synthesis on cytoplasmic ribosomes, mtTF1 must be efficiently targeted to and imported into the organelle. Once mtTF1 reaches the mitochondrial matrix, however, the job of finding its appropriate target sequence becomes quite simple; vertebrate mtDNA is a homogeneous population of 16-kilobase-pair closed circles. Nonspecific binding to a mtDNA molecule, followed by intramolecular transfer to a high-affinity binding site, would seem to be an efficient strategy for a mitochondrial transcriptional specificity factor. One of the classical procaryotic examples of this model is the *lac* repressor, which was also one of the first DNA-binding proteins to be subjected to quantitative analysis by the gel retardation technique (15). Although there is no similarity between their target DNA sequences and no evidence for any functional similarities between the two proteins, the behavior of mtTF1 reported here qualitatively resembles that reported by Fried and Crothers (15) for the *lac* repressor-operator interaction. In both cases, a ladder of shifted bands appeared with an increase in protein addition and seemed to represent complexes of a sequentially increasing protein-DNA ratios. Preliminary kinetic analysis further indicated that, as with the *lac* repressor, the stability of mtTF1-DNA complexes was inversely related to the number of factor molecules bound (Fig. 3; unpublished data).

A final similarity between mtTF1 and the *lac* repressor was the ability to saturate DNA fragments in the gel retardation assay. Fried and Crothers (15) detected eight discrete steps on their ladder, when they used the purified *lac* repressor and a 203-bp operator fragment, and reached apparent saturation at eight repressor tetramers per DNA molecule. This, they argued, was consistent with previous binding site measurements of 25 bp and with presumably complete coverage of DNA by protein. Purified mtTF1 also reached saturation after seven or eight incremental steps (Fig. 1), but with no clear proportional relationship to the length of the target fragment; fragments A and B (Fig. 1) were ~490 and ~270 bp, respectively, and showed, at most, a difference of one step in the ladder. Moreover, the mtTF1-binding site, which was defined by footprint boundaries, was approximately 23 bp; actual saturation of a 490-bp DNA fragment by eight molecules of mtTF1 would imply a significant occlusion of neighboring sites by factor binding and would be inconsistent with the saturation of a 270-bp fragment by seven molecules. Thus, we believe that the saturation phenomenon more likely reflects either a limit to resolution in the present gel system or an effect of protein-protein interactions intrinsic to mtTF1. One attractive model invokes high-density mtTF1 binding as a prerequisite for HSP stimulation. H-strand transcription reached maximal levels in vitro at higher mtTF1 to DNA ratios than were required for full LSP activity; HSP binding occurred only at similarly high protein to DNA ratios (Fig. 8). However, we cannot rule out the participation of additional, perhaps HSP-specific, factors that were depleted or denatured during purification in H-strand transcription in vivo. Similarly, we were unable to assess the significance of the minor subpopulations of mtTF1 that were separated from the main peak by MonoQ FPLC (Fig. 5A and D). We strongly suspect that these are variants (at least in chromatographic behavior) of the same protein, since they produced identical DNA mobility shifts; however, they showed an increased selectivity for HSP relative to the bulk form of the activity (Fig. 5A; unpublished data).

retardation) contained 400 μM ATP, 150 μM each of CTP and UTP, and 2 μM unlabeled GTP. The transcription reactions alone (A) each contained 16 μCi of [α-^{32}P]GTP and 4 μl of PC-purified mtRNA polymerase. The final KCl concentration was 20 mM in each case. Reaction volumes were 20 μl for transcription and footprinting and 10 μl for gel retardation. (A) Factor additions were none (lane 1), 0.06 μl (lane 2), 0.125 μl (lane 3), 0.25 μl (lane 4), 0.5 μl (lane 5), 1.0 μl (lane 6), 2.0 μl (lane 7), 3.0 μl (lane 8), and 4.0 μl (lane 9). Incubation time was 30 min. M, Marker. (B) The amounts of mtTF1 added were none (lanes 1 and 9), 0.125 μl (lane 2), 0.25 μl (lane 3), 0.5 μl (lane 4), 1.0 μl (lane 5), 2.0 μl (lane 6), 3.0 μl (lane 7), and 4.0 μl (lane 8). Following a 20-min incubation at 28°C, the fragments were digested with 0.5 μg of DNase I per ml for 30 s at room temperature. The asterisks indicate positions of enhanced DNase I cleavage flanking the mtTF1-responsive element of HSP. (C) The labeled fragments were incubated for 20 min at 28°C with no mtTF1 added (lane 1) and with 0.03 μl (lane 2), 0.06 μl (lane 3), 0.125 μl (lane 4), 0.25 μl (lane 5), 0.5 μl (lane 6), 1.0 μl (lane 7), 1.5 μl (lane 8), and 2.0 μl (lane 9) of MonoQ-purified mtTF1.

The notion that a common factor could recognize HSP and LSP through different binding modes or stoichiometries deserves some consideration in the light of the in vivo function of the two major promoters. In addition to its transcriptional role, LSP of vertebrate mtDNA is responsible for priming replication of the H strand (the leading strand in mtDNA synthesis) (8, 9), whereas HSP has no such dual function. On the other hand, the H strand encodes a preponderance of the genetic information that is resident in mtDNA, including the heavily transcribed rRNA genes (18). Thus, the ability of mtTF1 to bind the LSP upstream control element stably may reflect some requirement of the replication cycle, since transient binding, either at the HSP element or at mutated LSP elements, seems to be sufficient for at least some transcriptional stimulation (14).

Comparative studies of mitochondrial transcription factors from different species could resolve this issue. Specificity factors that can be dissociated from RNA polymerases have been reported in yeast (24, 29, 30, 35), *Xenopus* (5), and human mitochondrial systems. In addition, we have detected a transcriptional stimulatory activity in mouse mitochondrial protein fractions that resembles human mtTF1 in its chromatographic behavior (M. W. Gray, R. P. Fisher, and D. A. Clayton, unpublished data). In order to establish functional homology (if any exists) among these proteins, characterization at the genetic and immunologic levels may be required, as will functional studies. The overall mode of mtDNA transcription and replication and the displacement loop transcriptional control region itself have been conserved throughout vertebrate evolution; one might expect this conservation to be reflected in the transcriptional apparatus of mammalian and amphibian mitochondria. Yeast mtDNA, on the other hand, diverges markedly in its organization, expression, and maintenance; and the precise relationship between its transcription and replication has not been established. Close juxtaposition of promoter elements and origins of replication in yeast mtDNA has been inferred from sequence analysis of the mitochondrial genomes of petite mutants. Furthermore, mapping of the 5′ ends of transcripts and nascent DNA strands in these petite strains suggests a priming mechanism similar to that used by higher eucaryotes (2). Identification and characterization of an mtTF1 homolog of yeast mitochondria could reveal how the nucleus-encoded transcription machinery of the organelle has evolved in concert with its template.

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