DNA Wrapping and Bending by a Mitochondrial High Mobility Group-like Transcriptional Activator Protein*

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Robert P. Fisher†, Thomas Lisowsky‡, Melissa A. Parisi¶, and David A. Clayton
From the Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305-5427

Mitochondrial transcription factor 1 (mtTF1) is the only accessory protein known to be required for accurate and efficient promoter recognition by mammalian mitochondrial RNA polymerase. It activates transcription by binding immediately upstream of transcriptional start sites and shows an inherent flexibility in primary DNA sequence requirement. By application of a purification strategy designed for human and mouse mtTF1, a protein resembling mtTF1 was recently isolated from yeast mitochondria; its size (19 kDa), DNA-binding properties, and amino acid composition suggest identity to HM, a previously described abundant protein of yeast mitochondria. Both human and yeast proteins show a general ability to wrap or condense and unwind DNA in vitro and bend DNA at specific sequences. Recent determinations of the amino acid sequences of the human and yeast proteins reveal that both contain domains homologous to the nuclear high mobility group (HMG) proteins which have been implicated in diverse functions such as chromatin compaction and transcription stimulation. The ability to unwind and bend DNA may be fundamental to the documented roles of the mammalian protein in mitochondrial DNA transcription and replication priming and suggests a similar function for the yeast protein in yeast mitochondria.

Mechanistic studies of transcription initiation have revealed essential conservation of structure and function across wide evolutionary distances. Homologues have been demonstrated between nuclear trans-acting transcription factors and between the cis-acting control sequences that they recognize from species as divergent as yeast and humans (1). In contrast, transcription of mtDNA in different species has so far defied attempts at generalization. In mammalian evolution, barriers to cross-species heterologous mitochondrial transcription have arisen while promoter anatomy and the basic transcriptional apparatus have been conserved (2-6). The major transcriptional activator protein of mammalian mitochondria, mitochondrial transcription factor 1 (mtTF1), a 25-kDa factor that binds to upstream regulatory elements of mammalian mtDNA promoters, is flexible in its sequence recognition. In vitro heterologous transcription experiments implicate additional factors, or perhaps the mtRNA polymerase itself, as the most important determinant(s) of species-specific promoter recognition (7). When the mammalian mitochondrial transcription apparatus and its cognate promoters are compared with their yeast counterparts (or each other), little or no homology is apparent. In fact, transcription by the yeast mtRNA polymerase system seems to require only the minimal DNA sequence information contained within a necessary and sufficient nonanucleotide promoter encompassing the transcriptional start site (8).

Recently, we described the high-yield purification of human mtTF1 from mitochondria; this method was also used to identify an ~19-kDa protein in yeast mitochondrial extracts with very similar biochemical properties (9). The availability of human mtTF1 in large quantities led to the sequencing of a cDNA clone corresponding to the nuclear gene for this transcriptional regulator protein (10). Preliminary characterization of the putative yeast homologue of mtTF1 revealed it to be indistinguishable from the previously described HM, a basic protein with DNA-packaging capabilities characteristic of prokaryotic histone-like proteins (11, 12); hence we used the nomenclature p19/HM (9).

Concurrently, Diffley and Stillman (13) have determined by genetic analysis that ABF2, a yeast protein initially identified by virtue of its ability to bind preferentially to and alter the conformation of DNA containing autonomously replicating sequences (14), is a mitochondrial protein also likely to be identical with HM. ABF2 binds DNA both nonspecifically and specifically at sequences associated with both nuclear and mitochondrial replication origins (13-15). That ABF2 may play both regulatory and structural roles in the maintenance and expression of yeast mtDNA is suggested by the results of gene disruption experiments; elimination of ABF2 leads to slow growth on nonfermentable carbon sources, and to the loss of mtDNA in glucose media (13).

In this report, we show that human mtTF1, like yeast p19/HM/ABF2, can introduce negative supercoils into and bend DNA. Analysis of protein-DNA complexes formed at high mtTF1-DNA ratios reveals a pattern of DNase I protection that may reflect "phased" binding and which seems to expose preferentially to nuclease digestion sequences important for replication priming (16). In addition, human mtTF1 appears to recognize the yeast mtDNA promoter associated with the replication origin or5 (17), producing a DNase I footprint similar to that produced by ABF2 at the same DNA sequence.

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§ Supported by a postdoctoral fellowship from the science committee of NATO via the Deutsche Akademische Austauschdienst.
¶ Present address: Botanisches Institut I, Abteilung Morphologie und Cytologie, Universität Düsseldorf, D-4000 Düsseldorf 1, Germany.
|| Presently a Medical Scientist Training Program Trainee of the National Institute of General Medical Sciences Grant GM07365-16.

† The abbreviations used are: mtTF1, mitochondrial transcription factor 1; SDS, sodium dodecyl sulfate; LSF, light-strand promoter; HSP, heavy-strand promoter; bp, base pair(s); HMG, high mobility group.
**MATERIALS AND METHODS**

**Purification of DNA-binding Proteins**—Human mtTF1 was prepared either by sequential DEAE-Sephacel and phosphocellulose chromatography with a high salt-ionic strength gradient from isolated KB cell mitochondria, as previously described (18), or by lysis of isolated KB cell mitochondria in boiling SDS followed by hydroxylapatite chromatography in the presence of SDS (19), renaturation by excess Triton X-100 addition, and chromatography on phosphocellulose in the presence of Triton X-100 (9). Yeast plS/HM was purified from the triple protease-deficient strain of Saccharomyces cerevisiae, BJ2926, as the second method described above for human mtTF1. Mouse mtTF1 was purified through the phosphocellulose step, as described previously (7). All protein fractions used for in vitro assays were dialyzed against 100 mM Tris-Cl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol, 50% glycerol, with bovine serum albumin added to a final concentration of ~300 μg/ml, and stored at -20 °C. Gel purification, denaturation, and renaturation of mtTF1 were performed by the modification of the method of Hager and Burgess (20) as previously described (18).

**Plasmid Constructs Used to Assay DNA Binding**—The plasmid LacI fragment vector described by Brem et al. (21), pCY4, was used to tag fragment 1 with a mouse light-strand promoter (LSP) and generating plasmids suitable for binding site permutation. Single-stranded oligonucleotides corresponding to both strands of the human and mouse LSP were synthesized (Operon, Inc.), gel-purified, treated with polynucleotide kinase (Pharmacia LKB Biotechnology Inc.) in the presence of ATP, in the cold, and ligated to the plasmid pCY4 (kind gift of D. Galas, University of Southern California) that had been linearized at the Smal site of the polylinker and treated with calf intestinal alkaline phosophatase (Boehringer Mannheim). Ligation mixtures were used to transform Escherichia coli DH5 and clones containing single-copy insertions were identified by restriction enzyme analysis of miniprep DNA. The constructs pHLS-P57 and pMLSP-62 contain a single copy of the synthetic human (57 bp) or mouse (62 bp) LSP, respectively (see the orientation shown in Fig. 3A), flankcd by duplicated EcoRI-BamHI fragments of pBR322. The series of labeled fragments containing the insert permuted with respect to the fragment ends (see Fig. 3A) was generated by digestion with each of six restriction enzymes (BamHI, BstNI, EcoRI, EcoRV, HindIII, NheI), labeled with polynucleotide kinase and [γ-32P]ATP (Du Pont-New England Nuclear), and gel-isolated as previously described (18). These fragments and similar fragments derived from the vector pCY4 alone were then used in DNA binding assays with either human or mouse mtTF1 or yeast p19/HM, and the complexes formed were analyzed by nondenaturing gel electrophoresis. Conditions for DNA binding and electrophoresis were essentially as previously described (18), with factor additions as indicated in the legend to Fig. 3. The reactions were carried out in 20-μl total volume, however, and fragment 1 was always included, and unless otherwise noted, electrophoresis was used to normalize the radioactivity in each lane, and so correct for different labeling efficiencies at the different restriction sites. The exception is the BstNI-digested fragment of pHLS-P7; lanes containing this fragment have one-half the radioactivity of comparable lanes. Each fragment was incubated with an amount of DNA-binding protein titrated to maximize formation of protein-DNA complexes of 1:1 stoichiometry. In general, the quantity of protein needed to bind to DNA to produce the single shift (seen as complex 1) is ~10-50 ng of purified protein per ng of shifted linear DNA fragment. In this experiment as well as those of Figs. 6 and 7, the total mass of end-labeled fragment was ~2-4 ng, the protein added was 25-100 ng, and non-specific competitor inhibitor, poly(dI-dC), was added to a final reaction concentration of 0.5-2.0 μg/ml.

**RESULTS**

**Isolation of a Yeast Protein Similar to mtTF1**—When applied to yeast mitochondria, the mtTF1 purification scheme identified and purified essentially to homogeneity a single 19-kDa protein capable of binding DNA in a gel-retardation assay (9). The amino acid composition (Table I) of this polypeptide was virtually the same as that of a previously characterized 19-kDa DNA binding protein of yeast mitochondria, HM (11, 12). Comparison of the amino acid compositions of human mtTF1, the yeast 19-kDa DNA-binding protein (p19), and that previously reported for yeast HM (11,
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**FIG. 1. DNA-wrapping by human mtTF1 and yeast p19/HM in vitro.** A, relaxed closed circular plasmid DNA containing the human HSP and labeled with $^32$P was incubated with increasing amounts of purified human mtTF1 (lanes 2–7) or yeast p19/HM (lanes 8–10), in the presence of calf thymus DNA topoisomerase I. Introduction of supercoils generates the homogeneous, faster-migrating Form I DNA seen at intermediate concentrations (roughly 6–12 ng of mtTF1 per 2.5 ng of DNA) of both mitochondrial proteins (lanes 5, 6, and 9), whereas lower concentrations result in a heterogeneous population of topoisomers. Higher concentrations of both mtTF1 and p19/HM, in excess of the amounts needed for maximal supercoiling, cause a decrease in the number of supercoils introduced (lanes 7 and 10), an inhibitory effect which has been previously noted for yeast HM (11). Preparation of the labeled substrate generates additional bands whose migration in the gel is not affected by the topoisomerase I; these presumably represent nicked circular (Form 0) and linear (Form I) DNA. A, gel purification of human mtTF1.

**FIG. 2. Human mtTF1 introduces negative supercoils.** Populations of topoisomers, ranging from completely relaxed (lanes 1, with no mtTF1 added), to maximally supercoiled (lanes 6, with ~90 ng of human mtTF1 added to ~100 ng of DNA), were generated by calf thymus topoisomerase I and then electrophoresed in a 1% agarose gel either in the absence (A) or presence (B) of 1 µg/ml of the intercalating agent, chloroquine phosphate. Chloroquine intercalation results either in the introduction of positive supercoils in a relaxed or positively supercoiled molecule (B, lane 1), or in the removal of negative superhelical turns. As the mtTF1:DNA ratio increases, the positive supercoils introduced by chloroquine are titrated (B, lanes 2–6), indicating that the species seen in the control gel with increasing mtTF1 concentrations (A, lanes 2–6) contain increasing numbers of negative supercoils.

**TABLE I**

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<tr>
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</table>

$^a$ p19/HM purified in this work by sequential SDS-hydroxylapatite and phosphocellulose chromatography.

$^b$ HM amino acid composition according to Caron et al. (11).

$^c$ ND, not determined.

The table shows that the amino acid composition of human mtTF1 and yeast p19/HM proteins is similar, with a few differences. For example, human mtTF1 has a higher content of Cys and Asx compared to yeast p19/HM and HM.

12) showed all three to be similar. In particular, both the human and yeast proteins are rich in the basic residues lysine and arginine. This feature, together with its demonstrated ability to unwind relaxed closed circular DNA, formed the

B, or C (SDS-PAGE A, B, and C: lanes 7–12), both in the presence (+) or absence (−) of DNA topoisomerase I. Only fraction B, which contains the 25-kDa mtTF1 protein, is capable of packaging the DNA, leading to the generation of supercoiled forms by the nicking and closing topoisomerase I enzymatic activity.
basis for the classification of HM as a histone-like DNA-binding protein (23, 24). That HM, the 19-kDa protein, and the recently cloned ABF2 (13) are one and the same protein is suggested by their common properties, including the fact that the amino-terminal peptide sequence of the 19-kDa protein purified by denaturation-renaturation chromatography (KASKRTQLRNE-LIKQGKXRP) matches exactly that predicted by the ABF2 cDNA sequence (13).

**DNA Wrapping by Human mtTF1 and Yeast p19/HM.—**In the experiment shown in Fig. 1A a superhelical plasmid DNA was linearized, end-labeled with ³²P and re-ligated to yield a relaxed circular molecule (actually a population of closed circular molecules with superhelical densities distributed about zero). Incubation either with partially purified human mtTF1 (lanes 2–7) or with the yeast protein (lanes 8–10) in the presence of calf thymus topoisomerase I results in the introduction of superhelical turns into the relaxed circular DNA, suggestive of wrapping of the DNA about the binding protein(s) (25, 26). Supercolling is of sufficient magnitude to approach the upper limit of electrophoretic mobility represented by form I plasmid isolated from E. coli (data not shown), indicating the introduction of ~15 superhelical turns into the ~3000-bp plasmid. Maximal supercolling occurs at intermediate concentrations of both proteins; higher concentrations generate a heterogeneous population of more relaxed topoisomers indistinguishable from that formed at lower factor concentrations.

To establish that mtTF1, rather than some minor contaminating activity of similar mobility, was responsible for these conformational changes, we employed the SDS-polyacrylamide gel electrophoresis denaturation-renaturation scheme of Hager and Burgess (20). Gel-purified 25-kDa polypeptide (Fig. 1B, slice B) retains the DNA-packaging function as shown in Fig. 1C, lane 10. Parallel assays of DNA-binding, sequence-specific DNA-binding (27) (assayed by footprinting), and transcriptional activation have demonstrated that all of these activities reside in the 25-kDa mtTF1 (18).

**Mitochondrial TF1 Introduces Negative Superhelical Turns into Closed Circular DNA in the Presence of Topoisomerase I—**A transcriptional activator such as mtTF1 capable of significant conformational perturbations of DNA structure would be expected to induce negative superhelical turns, and thus promote underwinding and “opening” of the double helix (25, 28). The sign of the supercoils introduced by mtTF1 was determined by electrophoresing the DNA topoisomers generated in the presence of mtTF1 in agarose gels containing the intercalating agent, chloroquine phosphate (29). By increasing the number of base pairs per turn of covalently closed circular DNA, the binding of chloroquine necessitates an increase in the writhe of the DNA since the linking number is constant. This obligatory increase in writhe will have opposite effects on electrophoretic mobility of negatively and positively supercoiled DNA. The intercalation of chloroquine into DNA supercoiled by mtTF1 results in electrophoretic retardation of the DNA molecules, indicating that the superhelical turns introduced by mtTF1 are negative (Fig. 2). Similar results were obtained for yeast p19/HM (data not shown).

**Mammalian mtTF1 and Yeast p19/HM Bend Mitochondrial Promoter DNA—**The assignment of a possible structural role to mammalian mtTF1 raised the issue of whether the alteration of DNA topology by mtTF1-binding at high factor-to-DNA ratios in any way reflected the single binding event at the upstream control element of the mitochondrial LSP that is necessary and sufficient for transcriptional activation. To produce negative supercoiling detectable by electrophoretic analysis requires large amounts of mtTF1 or p19/HM. At the protein-to-DNA ratios resulting in maximal condensation, many factor molecules are complexed with each DNA molecule. In contrast, maximal stimulation of *in vitro* runoff transcription of LSP-containing fragments seems to require only a single binding event (18). To assess whether this sequence-specific binding altered promoter conformation, as first evaluated by Wu and Crothers (30), and to begin to address the question of whether such changes are involved in promoter activation, we employed the plasmid permutation vector system of Prentki et al. (21) to detect and map bends or kinks, either intrinsic or protein-induced, in the mammalian LSP (Fig. 3). Double-stranded oligonucleotides containing either mouse (62 bp) or human (57 bp) LSP sequences were inserted into a polylinker between tandem repeats of the 375-bp BamH1-EcoRI fragment of pBR322 (21) (Fig. 3A).

Permutation of the DNA sequences with respect to the ends of the fragment had no effect on the electrophoretic mobility of the free fragment, indicating that neither LSP is naturally bent. Binding of a single homologous mtTF1 molecule (or mtTF1 multimer) shifts the DNA to a slower mobility; the mobility of this complex (complex 1) varies greatly with sequence permutation for both mammalian LSPs, indicating that mtTF1-binding at the lowest detectable protein:DNA stoichiometry induces sequence-specific bending. While the position and magnitude of the bends appear similar in the two species, some differences are apparent. Only a single minimum of electrophoretic mobility is seen in the mouse system (Fig. 3C), whereas the human protein produces two minima, separated by a fragment with increased mobility (Fig. 3B). Both human and mouse mtTF1 bind and bend a control fragment, which is the permutation vector lacking an LSP insert. This vector contains sequences that might be expected to be cryptic mtTF1 flexible binding sites (7); this problem is common to available vector sequences. The mobility patterns for these control fragments differ between mouse and human, with the human having two minima and the mouse only one, as was the case for bending at target LSP sequences.

Bending of the control fragment unfortunately interferes with mapping these bends within the LSP, but allows us to dissociate bending from binding specificity. In Fig. 4 (lanes 1–12), the dissociation rates of mouse mtTF1 from the fragments containing or lacking the synthetic LSP were compared in the presence of excess competitor DNA. The mouse factor bends both fragments, but binds only the LSP-containing DNA with appreciable stability. The increased stability may explain the relatively greater difference in mobility between fastest and slowest migrating complexes seen with fragments containing the homologous LSP (see Fig. 3C, lanes 13–18 compared to lanes 1–6); however, a more likely explanation is that the natural target sequence undergoes more severe bending by the factor than does the cryptic bending site in the vector. We note that these bending patterns are dependent on the source of the factor (mouse or human) rather than the derivation of the DNA sequence; for instance, human mtTF1-mouse LSP complexes have two minima, while mouse mtTF1-human LSP complexes have one (Fig. 3, B and C).

Another experiment provided the first evidence that yeast p19/HM displays sequence preference in its interaction with DNA, and also suggested a possible connection with mitochondrial transcription. Like its putative mammalian homologue, mtTF1, yeast p19/HM induces bending of the control vector fragment lacking mtDNA sequences. However, the mobility minimum of the p19/HM-induced bend is displaced with respect to that associated with human mtTF1 (Fig. 3D, lanes 1–6). Insertion of the
FIG. 3. Bending of DNA by mtTF1 and p19/HM. A, construction of plasmid permutation vectors containing human and mouse mtDNA LSPs. Short, double-stranded oligonucleotides containing the LSPs of either human or murine mtDNA were cloned into the polylinker of plasmid pCY4 (21) at the Smal site. Digestion of the resulting plasmids with each of the six restriction enzymes with recognition sites within (or delimiting) the duplicated 375-bp EcoRI-BamHI sequence flanking the mtDNA insert effectively permutes the LSP from one end of the fragment to the other. Homologous mtTF1 binding sites are indicated by hatched boxes. B-D, permutation of the mtTF1-binding sites of the mammalian LSPs results in mtTF1-DNA complexes with different mobilities, indicative of fragment bending upon factor binding. Either human (B) or mouse (C) mtTF1, or yeast p19/HM (D) was incubated with labeled fragments derived from the vector pCY4 (lanes 1-6) or from plasmid permutation constructs containing the synthetic human (lanes 7-12) or mouse (lanes 13-18) LSP, by digestion with each of the six different restriction endonucleases that cleave within the duplicated sequence flanking the insert. The enzymes used were: EcoRI (lanes 1, 7, 13); HindIII (lanes 2, 8, 14); BstNI (lanes 3, 9, 15); EcoRV (lanes 4, 10, 16); NheI (lanes 5, 11, 17); and BamHI (lanes 6, 12, 18). Bending of the control fragment precludes a simple graphic summary of these data.

human LSP does not affect the position of bending (Fig. 3D, lanes 7-12). However, the introduction of the mouse LSP sequence causes a shift in the electrophoretic mobilities of the p19/HM-DNA complexes, indicating a bend in virtually the same location as the one caused by homologous murine mtTF1-binding (Fig. 3D, lanes 13-18 and Fig. 3C, lanes 13-18). This displacement of the bending center occurs without significant stabilization of the p19/HM-mouse LSP complex; the protein-DNA complexes containing the LSP dissociate as rapidly as do complexes formed on the vector control fragment (Fig. 4, compare lanes 13-18 with lanes 19-24), and p19/HM makes no DNase I footprint on this region of mouse mtDNA (data not shown). The "recognition" of the mouse LSP by yeast p19/HM thus consists of an unstable protein-induced bend resembling, by gel electrophoretic analysis, the conformation associated with homologous mtTF1-binding.

Human mtTF1 and Yeast p19/HM Bind mtDNA Regulatory Sequences—The LSP of mammalian mtDNA contains the strongest known binding site for mtTF1; like the promoter associated with yeast oris6 [and bound by ABF2 (13)] the LSP is an important element of a mtDNA replication origin. We have previously noted that mtTF1 can recognize and bind additional sites at the leading-strand origin region; this interaction occurs within a stretch of DNA containing signals for the processing of RNA transcripts to produce primers for replication and may depend on the presence of multiple repeats of a sequence element also found within the LSP (22). Using labeled DNA probes containing sequences from this
region of the human leading-strand replication origin, we have analyzed this binding by mtTF1 more carefully (Fig. 5). Incremental addition of mtTF1 molecules to origin-containing DNA fragments leads to the stepwise retardation of the fragments' migration in nondenaturing gels. Saturation appears to occur after seven or eight steps, resulting in a complex that cannot be retarded further (Fig. 5, band pattern of mtTF1 binding at different stoichiometries (Fig. 5, D). DNase I digestion of these complexes prior to their resolution in the nondenaturing gel allows us to analyze the stepwise retardation of the DNA fragments' migration in nondenaturing gels. Saturation appears to occur after seven or eight steps, resulting in a complex that cannot be retarded further (Fig. 5, band pattern of mtTF1 binding at different stoichiometries (Fig. 5, D). DNase I digestion of these complexes prior to their resolution in the nondenaturing gel allows us to analyze the pattern of mtTF1 binding at different stoichiometries (Fig. 5, lanes A and C). Periodic discrete binding is clearly apparent only in the “saturated” complexes, suggesting that high density binding is required for “packaging” of the DNA into a well-defined structure. Within this structure, known sites of replication origin?-When large numbers of mtTF1 molecules bind to a mtDNA fragment containing the origin of human leading-strand synthesis, sequences implicated as RNA processing sites and guide elements for the activity of RNase MRP (16) remain exposed to the endonucleolytic activity of DNase I (Fig. 5, lower panels). This observation raises the intriguing possibility that mtTF1 may play a role in organizing the replication origin for proper mtDNA replication priming. A DNA organizational role has been proposed for ABF2 in yeast mitochondria, on the basis of comparable experiments (13, 15). One advantage of this model is that it explains how an abundant structural protein capable of packaging the bulk of the yeast mitochondrial genome might also exert a regulatory effect on transcription and/or replication, as is suggested by genetic studies (13). In the case of mammalian mtTF1, which has a well-documented transcriptional regulatory role but does not appear to be as abundant as ABF2 (9), the mitochondrial genome-organizing function may be restricted to the displacement-loop region, which contains the leading-strand replication origin as well as both major transcriptional promoters of mammalian mtDNA. We have previously shown a differential response of the human LSP and heavy-strand promoter (HSP) to varying mtTF1:DNA ratios; the HSP is activated in vitro only when complexed with multiple mtTF1 molecules, while LSP activation requires only a single binding event (18). Thus the conformational changes effected by mtTF1 may play a direct part in differential regulation of

**DISCUSSION**

A Role for mtTF1 and p19/HM/ABF2 in mtDNA Replication?—When large numbers of mtTF1 molecules bind to a mtDNA fragment containing the origin of human leading-strand synthesis, sequences implicated as RNA processing sites and guide elements for the activity of RNase MRP (16) remain exposed to the endonucleolytic activity of DNase I (Fig. 5, lower panels). This observation raises the intriguing possibility that mtTF1 may play a role in organizing the replication origin for proper mtDNA replication priming. A DNA organizational role has been proposed for ABF2 in yeast mitochondria, on the basis of comparable experiments (13, 15). One advantage of this model is that it explains how an abundant structural protein capable of packaging the bulk of the yeast mitochondrial genome might also exert a regulatory effect on transcription and/or replication, as is suggested by genetic studies (13). In the case of mammalian mtTF1, which has a well-documented transcriptional regulatory role but does not appear to be as abundant as ABF2 (9), the mitochondrial genome-organizing function may be restricted to the displacement-loop region, which contains the leading-strand replication origin as well as both major transcriptional promoters of mammalian mtDNA. We have previously shown a differential response of the human LSP and heavy-strand promoter (HSP) to varying mtTF1:DNA ratios; the HSP is activated in vitro only when complexed with multiple mtTF1 molecules, while LSP activation requires only a single binding event (18). Thus the conformational changes effected by mtTF1 may play a direct part in differential regulation of

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**Fig. 4. DNA bending does not require stable binding.** Mouse mtTF1 (lanes 1-12) or yeast p19/HM (lanes 13-24) were preincubated with the NheI-NheI fragment of pMLSP (lanes 1-6 and 13-18) or of the vector pCY4 (lanes 7-12 and 19-24) for 20 min at 28 °C in a final volume of 80 μl. Aliquots (10 μl) of each reaction mixture were loaded on a running 4% polyacrylamide nondenaturing gel (lanes 1, 7, 13, and 19) before a ~100-fold mass excess of pMLSP circular plasmid DNA was added to the remainder. The samples were then incubated at 28 °C, and at the times indicated below each lane, aliquots were withdrawn and loaded. Introduction of the mouse LSP, with its associated mtTF1-binding site, is required for the formation of kinetically stable complexes between the labeled fragment and mouse mtTF1. Similar results have been obtained with human mtTF1 (18). Complexes between mouse mtTF1 and the vector pCY4 fragment dissociate virtually instantaneously upon addition of competitor DNA (compare lanes 7 and 8). Thus our ability to detect bending of this fragment by mouse mtTF1 implies that the factor may influence DNA conformation both at transcriptional promoters and by transient binding within nonpromoter DNA. The yeast protein p19/HM resembles mtTF1 in its ability to bend DNA without binding stably to it; comparison of the dissociation kinetics of p19/HM-DNA complexes with fragments containing (lanes 13-18) or lacking (lanes 19-24) the synthetic mouse LSP further demonstrate that a change in bending center preference (see Fig. 3D) can occur without an enhancement of binding stability.
FIG. 5. DNase I protection analysis of mtTF1-DNA complexes resolved by gel electrophoresis. Preparative DNA binding reactions were carried out with ~8 ng of a BamHI-EcoRI fragment derived from deletion clone L5'Δ+1, 32P-labeled at either the 5' or 3' end. Reactions contained low (2 μl, lanes A and C), or high (16 μl, lanes B and D) concentrations of mtTF1, and were performed exactly as previously described for LSP-containing fragments (18). After preincubation, the DNA-protein mixture was digested with DNase I and electrophoresed on non-denaturing 4% polyacrylamide gels. The gel was fractionated using the autoradiogram shown at the top to locate and excise mtTF1 complexes 1–3, as well as the saturated complex (S) and uncomplexed free fragment (ff). The DNA was eluted from these gel slices, precipitated with ethanol, denatured, and electrophoresed in 6% polyacrylamide sequencing gels shown at the bottom. The lane designations correspond to sequencing ladders, free fragments, or the complex from which the DNA was extracted. The numbers to the left of each panel refer to the published sequences of human mtDNA (22, 31). The hatched bars indicate the sequences protected by mtTF1 binding, while the open bars denote the conserved sequence blocks I–III, which are apparently largely excluded from mtTF1 binding.

FIG. 6. Recognition of yeast mtDNA sequences by human mtTF1. A fragment of yeast mtDNA from the ori5 region was labeled on either strand at the same site and incubated with increasing amounts of human mtTF1, as indicated below each lane. The footprint produced by human mtTF1 is indicated by the hatched bars alongside the sequence ladders, and arrows indicate the start site and direction of transcription by the yeast transcriptional apparatus (34, 35). The footprint produced by yeast p19/HM/ABF2 on the yeast ori5 sequence appears to contact nucleotides slightly further upstream of the transcriptional start site (13); thus the footprints produced by the human and yeast proteins are similar, but not identical.

transcription and, in turn, replication priming from the LSP (36). In any case, it seems likely that the unusually high affinity of this sequence-specific transcriptional activator protein for random, nonpromoter DNA, a paradox that has been evident virtually from the first description of mtTF1, reflects this structural role.

Wrapping and Bending in the Regulation of Mitochondrial Transcription—The results described above establish that mtTF1 can induce significant conformational changes in DNA upon binding. These changes include wrapping of the DNA, presumably about multiple mtTF1 molecules in an essentially nonspecific protein-DNA complex, and bending of DNA by a single mtTF1 molecule at specific sequences, including the mammalian LSP.

While both mtTF1 and p19/HM/ABF2 resemble E. coli HU protein and its prokaryotic homologues in their amino acid composition and ability to condense DNA by introducing negative superhelical turns, they appear to be more related structurally to the eukaryotic HMG proteins, as recent molecular analyses have shown (10, 13). These proteins, long suspected of influencing gene expression by altering DNA conformation, have now been directly implicated in the control of transcription by nuclear RNA polymerase I (37) and have been suggested to play a role in RNA polymerase II transcription as well (38). To date, no exact mechanism for sequence-specific DNA binding by this class of proteins has been deduced; the HMG proteins apparently contain a new type of DNA-binding structural motif. The ability to produce
large quantities of both mtTF1 and p19/HM/ABF2 in vitro from bacterial expression vectors should facilitate structural analysis; the mitochondrial factors are attractive candidates for such an analysis because of their small size and shared ability to bind DNA both specifically and nonspecifically.

**p19/HM/ABF2 and mtTF1; a Link between Yeast and Mammalian Mitochondrial Transcription?**—While biochemical similarities among mammalian, amphibian, and yeast mtRNA polymerases have been reported (6, 34, 39–43), the mechanisms of promoter recognition, and in particular the modes of action of the major transcriptional specificity factors that have been identified, appear to be quite different in the different classes of eukaryotes. In *S. cerevisiae* a 145-kDa mtRNA polymerase and a 43-kDa specificity factor, both isolated from polyacrylamide gel slices, have been reported to bind, bend, and initiate transcription correctly from the simple nonanucleotide promoter of yeast mtDNA in vitro (34, 35). Mammalian mitochondrial promoters, in contrast, have a more complicated structure. Upstream regulatory elements, 12–39 bp from the transcriptional start sites, modulate transcription levels through the action of mtTF1. Evidence for even greater complexity comes in mammalian systems from...
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**Fig. 8.** Sequence alignment of human mtTF1 and yeast ABF2 (p19/HM). Top, the basic organization of human mtTF1 (h-mtTF1) (10) and yeast ABF2 (13) proteins is shown. The *hatched regions* denote putative leader sequences for import into the mitochondrial matrix, HMG boxes are shaded and denoted as box 1 and box 2, and the *numbers* refer to amino acids with number 1 defined as the amino terminus of the mature protein isolated from mitochondria. *Bottom,* amino acid sequence alignment of h-mtTF1 (10) and ABF2 (13) is shown. Identical amino acids are connected by a *vertical bar;* conservative amino acid substitutions are indicated by a *dotted line.* In this alignment, the size difference between the two proteins is largely due to the absence of yeast sequence between the HMG boxes and at the carboxyl terminus. Identity in the directly compared amino acids is 21% and similarity is 93%, based on grouping conserved amino acids as follows: (P, G), (S, T), (Q, N), (E, D), (K, R), (M, C), (V, L, I, A), (F, Y, W, H).

**in vitro** analyses that imply the existence of an additional cis-acting element in mouse mtDNA (4), and possibly an additional trans-acting factor in human mitochondrial extracts (39), and in yeast from genetic studies that identify multiple nuclear loci that regulate mitochondrial transcription (44, 45).

The cDNA sequences of human mtTF1 (10) and ABF2 (13), together with the *in vitro* comparisons of the proteins reported here, show that the two proteins are similarly structured and raise the possibility that they are indeed functional homologues. The question whether or not a specific function is the "housekeeping" function of yeast ABF2, or a general function such as the "housekeeping" function of yeast ABF2, is largely due to the absence of yeast sequence between the HMG boxes and at the carboxyl terminus. Identity in the directly compared amino acids is 21% and similarity is 93%, based on grouping conserved amino acids as follows: (P, G), (S, T), (Q, N), (E, D), (K, R), (M, C), (V, L, I, A), (F, Y, W, H).

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This question may remain unresolved until an exact role for p19/HM/ABF2 in activating mitochondrial transcription in yeast is established or excluded. On the other hand, this report and that of Diffley and Stillman (15) suggest a role for this newly discovered class of mitochondrial HMG-like proteins in the three-dimensional organization and replication of the genome. Thus the two proteins may have overlapping, rather than identical functions. A useful paradigm may be the histone-like proteins of prokaryotes, to which HM was originally thought to be homologous (11, 12). These proteins are interchangeable to a limited extent, but each member of the class shows distinct adaptations (such as sequence-specificity on the part of integration host factor) to its primary role (23, 24, 46). Elucidation of the binding mechanisms employed by p19/HM/ABF2 and mtTF1 in both their specific and nonspecific interactions with mtDNA may well uncover novel protein-nucleic acid associations.

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3 B. Xu and D. A. Clayton, unpublished observations.
REFERENCES