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PREFERENCE OF HUMAN MITOCHONDRIAL RNA POLYMERASE FOR SUPERHELICAL TEMPLATES WITH MITOCHONDRIAL PROMOTERS

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The RNA polymerase of HeLa cell mitochondria has been purified free of endonuclease and DNA topoisomerase activities, permitting evaluation of the effect of template topology on transcription in vitro. On single-stranded DNA templates, transcription is nonspecific and does not require mitochondrial DNA sequences. In contrast, duplex DNA templates are efficiently transcribed only when they (1) carry the mitochondrial D-loop region and (2) are negatively supercoiled. These findings suggest a role for template superhelicity in modulating mitochondrial transcription in vivo.

Changes in the degree of negative supercoiling of DNA templates influence the in vitro activity of RNA polymerases from various prokaryotic (1,2) and eukaryotic nuclear systems (3,4). It has been shown that DNA topoisomerases are involved in the regulation of gene expression in vivo through the use of specific inhibitors and mutants (reviewed in ref. 5). Although a significant portion of the mitochondrial DNA (mtDNA) isolated from mammalian cells is negatively supercoiled (6), the role that this negative-superhelicity might play in controlling mitochondrial (mt) transcription is unknown. Partially-purified fractions of human mt RNA polymerase have been shown to recognize mt promoters in either linear or relaxed circular duplex templates, initiating transcription specifically in vitro at the same sites that are used in vivo (7,8,9,10). However, nuclease or DNA topoisomerase contamination of various mt RNA polymerase preparations (7,8,10) has prevented the analysis of the effect of template superhelicity on transcription activity.

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Abbreviations: mt, mitochondrial; PMSF, phenylmethylsufonylfluoride; DTT, dithiothreitol; BSA, bovine serum albumin; ss, single-stranded; ds, double-stranded; D-loop, displacemnt loop.

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We have prepared human mt RNA polymerase free of DNA topoisomerase and nuclease activities and have investigated the transcriptional activity of this enzyme on templates of various sequences and degrees of superhelicity. This RNA polymerase fraction shows significant transcriptional activity on duplex templates which are negatively supercoiled, and include the heavy(H) and light(L)-strand mtDNA promoter sequences. However, almost no activity is found with templates which either are relaxed or lack mt promoter DNA. These *in vitro* results suggest that alterations of torsional strain in the mt genome may be involved in the regulation of mt transcription *in vivo*.

MATERIALS AND METHODS

Isolation of mitochondria

Fresh HeLa cells were obtained from the Tissue Culture Support Center at the Washington University School of Medicine. About 40 g of wet packed cells were washed in phosphate buffered saline and resuspended in 100 ml of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 1.5 mM $CaCl_2$. Mitochondria were isolated immediately by the two step gradient method of Bogenhagen and Clayton (6), without mannitol and with Ca^{++} in place of Mg^{++} in the swelling buffer.

Enzyme purification

All steps were carried out at 0-4°C. Isolated mitochondria from 40 g of HeLa cells were thawed and 1 mM PMSF, 1 mM DTT, and 0.4 M KCl were added. Lysis was achieved by sonication (Fraction I; see Table I); the lysate was then clarified at 25,000 xg for 30 min (Fraction II). This fraction was diluted two-fold with buffer A (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10% glycerol) and centrifuged at 145,000 xg for 120 min. The gel-like pellet (Fraction III) containing 80-90% of the RNA polymerase activity was resuspended by gentle Dounce homogenization in 2.0 ml of buffer A plus 1 M NaCl and recentrifuged at 145,000 xg for 120 min. The RNA polymerase activity was then recovered in the supernatant (Fraction IV). It was concentrated by precipitation using 0.3 g $(NH_4)_2SO_4$ per ml and redissolved in 0.1 ml of buffer A plus 0.2 M KCl (Fraction V). The $(NH_4)_2SO_4$ concentrate was applied to an 8 ml Sephacryl S-300 column equilibrated with buffer A plus 0.2 M KCl. The RNA polymerase activity eluted in a single, symmetrical peak (Fraction VI). Active fractions pooled from two such S-300 columns were combined and applied to a 0.2 ml phosphocellulose column (Whatman P11) equilibrated in buffer A plus 0.2 M KCl, washed with 2 ml of buffer A plus 0.3 M KCl, and eluted with buffer A plus 0.6 M KCl. Peak fractions were pooled (Fraction VII), frozen in liquid N_2 and stored at -80°C. No significant activity was lost after 18 months, despite repeated freezing and thawing.

Template DNAs

The mt L-strand origin of replication (Ori_L) was cloned into the bacteriophage vector M13mp9 via the EcoRI and HindIII sites at nucleotide positions 5274 and 6203, respectively, of the human mtDNA sequence (11), creating the recombinant phage $M13mtOri_L$. Recombinant phage $M13mtOri_H$ was a gift from Dr. Ray Monnat (University of Washington) and contains the 1.2 kb fragment of human mtDNA from a SacI site (position 36) to an XbaI site (position 1193) which encodes the two mt promotors and the H-strand origin of replication (Ori_H); this was inserted into the XbaI, SacI region of vector M13mp11. In both recombinant phages, the orientation is such that the mt H-strand is linked with the viral (+) strand. To construct the plasmid $pXfmtOri_H$, the mt insert was removed from the M13 phage DNA using the HindIII and EcoRI sites flanking the XbaI and SacI sites, and religated into plasmid pXf3 (12). Plasmid DNAs and replicative forms (RF_I) of the

M13 phages were prepared as outlined (13). Viral DNA was purified by phenol extraction of phage particles and ethanol precipitation.

Partially relaxed M13mtOri$_H$ DNA was prepared by incubating 14 µg of supercoiled RF$_I$ DNA for 30 min at 37°C in 250 µl of: 50 mM Hepes (pH 7.8), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 3% (w/v) glycerol, 0.5 mM EDTA, and 2-24 units of E. coli DNA topoisomerase I (purified as in ref. 14), or 500 units of HeLa DNA topoisomerase I (purified as in ref. 15). The DNAs were then extracted with phenol/chloroform (1:1) followed by chloroform alone, and ethanol precipitated. Extent of relaxation was assessed by agarose gel electrophoresis.

RNA polymerase assay

Each reaction mixture contained in 50 µl: 10 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.1 mg/ml BSA, 1 mM ATP, 1 mM DTT, 150 µM each of GTP and CTP, 60 µM [^{3}H]UTP (300 cpm/pmol) and 50 µg/ml of M13mtOri$_L$ ssDNA. Reactions were incubated 30 min at 37°C and terminated by acid precipitation. One unit of mt RNA polymerase activity promotes the incorporation of 1 pmol of UTP into acid insoluble material in 30 min at 37°C.

Other assays

Protein concentration was determined by the method of Bradford (16), using BSA as a standard. DNA topoisomerase I and endonuclease assays were carried out under conditions identical to the RNA polymerase assay except that the total volume was 20 µl, phage RF$_I$ DNA at 12 µg/ml was substituted for the RNA polymerase template, and ribonucleotides were omitted. One unit of endonuclease converts 50% of the input RF$_I$ DNA to the nicked form II in 30 min at 37°C.

RESULTS

Partial purification of RNA polymerase and separation from DNA topoisomerase

Using an assay that measures the ssDNA-directed incorporation of ribonucleoside triphosphates into acid-insoluble products, the RNA polymerase activity identified in lysates of HeLa cell mitochondria was purified 300-fold with an 8% yield (Table 1). This RNA polymerase activity was considered to be mitochondrial because it was the only RNA polymerase activity detected in isolated mitochondria, and because its biochemical and physical properties as listed in Table 2 are similar to the properties of mt RNA polymerases from other systems and are significantly different from the properties of the nuclear RNA polymerases (7,10,17-21). The complex requirements for Mg^{++} and Mn^{++} shown in Fig. 1 are also typical of the

Table 1. Purification of Mitochondrial RNA Polymerase

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification (-fold)
I. Mitochondrial lysate	46	16,600	360	100	1
II. Cleared lysate	23	9,000	390	54	1.1
III. Low salt pellet	9.5	11,700	1,200	71	3.3
IV. High salt supernatant	2.6	11,900	4,700	72	13
V. $(NH_4)_2SO_4$ precipitate	1.2	7,300	6,100	44	17
VI. Sephacryl S-300	0.17	3,800	22,000	23	61
VII. Phosphocellulose*	0.024	3,300	142,000	8	390

*Data from two combined S-300 fractions.

Table 2. Biochemical and Physical Properties

Activity in presence of KCl (20 mM)	65%
(100 mM)	36%
" Rifampicin (1 μg/ml)	96%
(100 μg/ml)	96%
" α-amantin (1 μg/ml)	91%
(100 μg/ml)	94%
" N-ethylmaleimide (1 mM)	1%
Sedimentation rate ($s_{20,w}$), from glycerol gradient	7.5S
Stokes radius, from S-300 column	42 Å
Native molecular weight (calculated)	130,000

mt RNA polymerase, whereas the nuclear enzymes either prefer Mn^{++} or use Mn^{++} and Mg^{++} equally well (22). In addition, the RNA polymerase activity was initially found in an insoluble form (Fraction III), as were mt RNA polymerases in other systems (20,23).

After solubilization and gel filtration, the RNA polymerase fraction still contained DNA topoisomerase I and endonuclease. Subsequent chromatography on phosphocellulose removed essentially all of those activities, with only a negligible amount of endonuclease remaining. Less than 0.05 units of either DNA endonuclease or DNA topoisomerase I were present per 4.0 units of Fraction VII.

Selectivity for duplex DNA which contains the mt D-loop region

As is true of many RNA polymerases (24), the HeLa mt RNA polymerase was more active on ssDNA than on dsDNA at all stages of purification (shown in Figure 2 for Fraction VII). On dsDNA, the initial fractions of the mt RNA polymerase showed almost no activity and did not distinguish between templates with or without mt DNA sequences (data not shown). However, Fraction VII enzyme did show significant activity on M13mtOri$_H$ ds DNA (42 units/μg enzyme), while still showing almost no incorporation with the vector M13mp9 DNA (Fig. 2). Under the same conditions *E. coli* RNA polymerase showed similar activity on both M13mtOri$_H$ and M13mp9, indicating that the preferential activity of the mt RNA polymerase on the Ori$_H$-containing plasmid is a specific characteristic of the mt enzyme. When the same mt D-loop region fragment was recloned into the duplex plasmid vector pXf3 (a derivative of pBR322), the mt enzyme again showed more activity with the recombinant plasmid than with the vector DNA alone (data not shown).

Three different methods were used to try to detect specific initiation of transcripts at the mt promotors with Fraction VII: run-off transcription of linearized templates, S1 mapping of transcripts from supercoiled circular templates, and hybridization of transcripts to restriction fragments of the template. A large amount of non-specific transcription was seen in all cases; however, no specific initiation was detected (data not shown). Thus, mt RNA polymerase Fraction VII is stimulated by the presence of D-loop

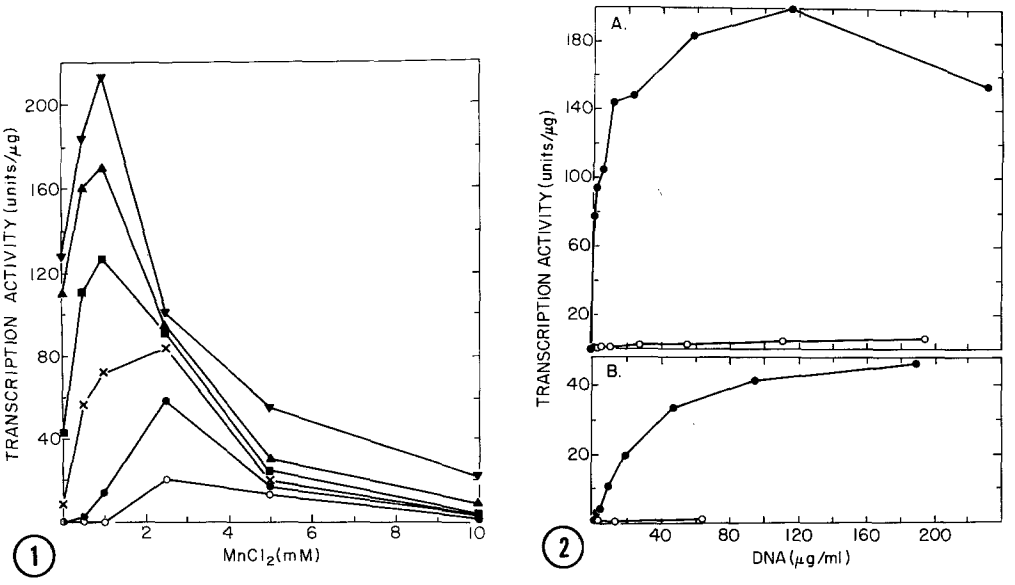

Figure 1. Cation requirement of the mt RNA polymerase. An 0.08 μg sample of Fraction VII was assayed in the presence of varying amounts of $MgCl_2$ or $MnCl_2$ or both, as indicated: o, no $MgCl_2$; •, 2.5 mM $MgCl_2$; X, 5 mM $MgCl_2$; ■, 10 mM $MgCl_2$; ▲, 20 mM $MgCl_2$; ▼, 30 mM $MgCl_2$. Assay conditions were otherwise as described in Materials and Methods.

Figure 2. Template requirement of the mt RNA polymerase. Samples of Fraction VII, 0.005 μg each, were assayed using the indicated concentrations of various phage DNA templates. Reactions contained 30 mM $MgCl_2$, 1 mM $MnCl_2$ but were otherwise as described in Materials and Methods. A. •, ss M13mtOri$_L$; o, ds M13mtOri$_L$. B. •, ds M13mtOri$_H$; o, ds M13mp9 (no mtDNA).

region DNA in a duplex template but initiation is apparently not limited to a single, unique, site.

Importance of negative superhelicity for activity on a physiological template

During the purification of mt RNA polymerase, the appearance of activity on duplex templates coincided with the removal of DNA topoisomerase and endonuclease activities. This coincidence suggested that superhelicity might be important for transcription on duplex templates. In order to test this proposal, the mt RNA polymerase was assayed on a set of progressively relaxed duplex templates. As shown in Figure 3, when the template was relaxed just enough to slightly retard its migration on an agarose gel compared to the naturally occurring RF_I species, the transcriptional activity of the mt RNA polymerase was decreased more than four-fold (compare lanes 1 and 2 with lanes 3 and 4). Further relaxation of the template resulted in even lower transcriptional activity (remaining lanes). No transcription was detected on a similar set of topoisomers of M13mp9 RF_I DNA (less than 1 transcriptional unit/μg enzyme; data not shown).

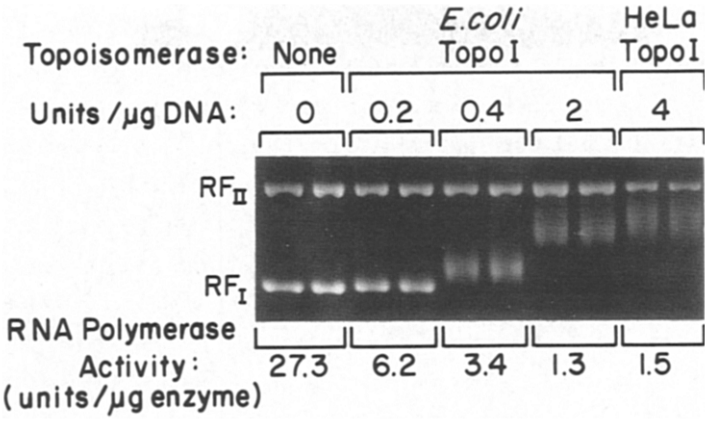

Figure 3. Effect of superhelicity on template activity. M13mtOri$_H$ RF DNA was relaxed to different extents with E. coli or HeLa cell DNA topoisomerase I. The resulting topoisomers were purified and used as templates for mt RNA polymerase. Each 56 µl reaction mixture contained the template DNA at 45 µg/ml, 0.12 µg Fraction VII RNA polymerase, 30 mM $MgCl_2$ and 1 mM $MnCl_2$; conditions were otherwise as described in Materials and Methods. After 30 min at 37°C, 5 µl of each reaction was examined on a 0.8% agarose gel (shown), and the remainder of each reaction was acid precipitated to determine transcription activity . Each reaction was performed in duplicate as indicated here by the paired lanes of the gel. The source and amount of the DNA topoisomerase used to prepare each template is indicated at the top of the gel; the average transcription activity of the duplicate reactions is indicated below. RF_I and RF_{II} designate the supercoiled replicative and nicked phage form DNA's, respectively; bands in between are particularly relaxed topoisomers.

Potential secondary structure in mt D-loop recombinants

The cloned fragment of mtDNA in M13mtOri$_H$ includes the entire $tRNA^{phe}$ gene and approximately one half of the 12S rRNA gene, both of which have the potential to form extensive secondary structures and thus might affect local "openness" of the template DNA. However, a subclone of the D-loop fragment (in pXf3) which includes only the $tRNA^{phe}$ gene and a portion of the 12S gene, showed no template activity. Therefore, it is unlikely that the potential secondary structure in this particular region non-specifically stimulated the RNA polymerase on the pXfmtOri$_H$ or M13mtOri$_H$ templates.

DISCUSSION

Mammalian mitochondria contain a circular duplex DNA genome on which a single major promoter has been identified for each strand (25). The H- and L-strand promoters are both located just upstream from the origin of H-strand DNA replication (Ori$_H$), near one end of the displacement loop ("D-loop") region.

Control of the DNA topology in this region may be an important mechanism for regulating transcriptional activity. In the present study, we have shown that extensive transcription in vitro by the human mt RNA polymerase

on ds templates is strongly dependent on negative supercoiling. Even a modest reduction in template superhelicity significantly decreased total transcription activity. Such effects are well-established for non-mitochondrial RNA polymerase activities *in vitro* (1,2,4) and *in vivo* (5). It is interesting that our final fraction of the human mt RNA polymerase does not show the specific initiation at the H- and L-strand promoter loci that has been observed with other mt RNA polymerase preparations (8,9,10). This result might be expected given the data of Fisher and Clayton which shows that a specificity factor can be separated from the mt RNA polymerase by chromatography on phosphocellulose (26). However, our enzyme still shows a strong preference for templates containing the mt promotors. Hence, the mt RNA polymerase "core" enzyme may have an intrinsic ability to recognize the D-loop region even if one or more accessory factors are required to limit initiation to the mt promoters; that is, specific initiation of transcription likely involves multiple, independent events. Evidence exists in the mouse mt system for DNA sequence domains that influence the formation of a "preinitiation complex" independent of elongation (27), and for transcription initiation at new sites when the normal start site is deleted from DNA templates (28). The human mt promotors have also been shown to have domains that influence the efficiency of transcription (8). Work is currently in progress to identify which domains of the human mt D-loop region elicit this transcriptional activity of the core enzyme on supercoiled DNA templates.

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