A study on the human mitochondrial RNA polymerase activity points to existence of a transcription factor B-like protein

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Abstract In the present work, the RNA polymerase activity of the human mitochondrial RNA polymerase mature protein (h-mtRPOLm) is shown, and its molecular activity calculated (2.1 ± 0.9 min^{-1}). An activity analysis of h-mtRPOLm and deleted versions of it has demonstrated that the entire recombinant protein is required for this activity. In addition, h-mtRPOLm alone or in presence of the known mitochondrial transcription factors (human mitochondrial transcription factor A and/or human mitochondrial transcription termination factor) is not able to initiate transcription from the specific human mitochondrial promoters pointing to the existence of a human mitochondrial transcription factor B-like protein.

Key words: Mitochondrial transcription; Mitochondrial RNA polymerase; Mitochondrial transcription factor A; Mitochondrial transcription factor B; Mitochondrial transcription termination factor

1. Introduction

Mitochondria are energy-producing organelles that contain multiple copies of a genome, the mitochondrial DNA (mtDNA) [1]. Human mtDNA transcription initiation sites and promoter regions have been determined. The mitochondrial genome is transcribed from one heavy strand promoter (HLP) with two initiation sites (H_1 and H_2) and one light strand promoter (LSP) with one initiation site (L) [2,3]. This genome contains binding regions for human mitochondrial transcription factor A (h-mtTFA) [4] and human mitochondrial transcription termination factor (h-mTERF) [5]. In vitro transcription initiation requires a mitochondrial RNA polymerase (h-mtRPOL) fraction and h-mtTFA [6]. However, in yeast and frog the mtRPOL initiate specific and accurate transcription aided by other transcription factor, mtTFB [7,8]. Thus, whether human transcription initiation complex consists of just h-mtRPOL and h-mtTFA or that it requires additional factors remains an important question [9].

The availability of human mitochondrial cDNAs encoding mtRPOL [10], mtTFA [11] and mTERF [12] provide the possibility to study the transcription system in vitro. The present work shows that h-mtRPOL, despite to an non-specific RNA polymerase activity, is not able to initiate specific transcription in vitro, and other factor(s) different from h-mtTFA and h-mTERF must be necessary for initiation.

2. Materials and methods

2.1. Plasmid constructs and in vitro expression of proteins

The h-mtRPOL cDNA was subcloned in an in vitro expression vector (pSPUTK, Stratagene), without the sequence that encodes the mitochondrial target sequence, corresponding at the first 41 amino acids [10]. The sequence encoding the mature protein was PCR-amplified using a forward primer matching the sequence of the desired 5′ end with a *Nco*I site (CC ATG GCC GCC AGC CCC CAG GAG C-177, which produce a substitution of amino acids SS(+43) for MA), and a reverse primer matching the sequence of the desired 3′ end with the *Sal*I site (GTC GAC AAG GCT CAC GGG GTG TCA-3723). The PCR fragments and pSPUTK vector were digested with *Nco*I and *Sal*I, ligated and finally transformed in INVαF′ cells (Invitrogen). H-mtTFA cDNA was subcloned in a pSPUTK vector without the first 42 amino acids corresponding to the target peptide [11], using the same strategy described above: forward primer, CC ATG GCT GTC TTG GCA AGT TGT C-280, that produce the substitution of SS (+44) for MA, and reverse primer, GTC GAC TTA ACA CTC CTC AGC ACC-856. The construction of mature h-mTERF was described previously [12]. The h-mtRPOL mutants lacking a segment at the 5′ and/or 3′-ends (ΔN406, ΔC100, ΔC400, ΔNC2 and ΔNC3) were PCR-amplified from the plasmid containing the mature h-mtRPOL by using the appropriate combination of primers matching the sequence with the desired 5′ and 3′-ends. The correct nucleotide sequence of constructs was verified by chain terminator sequencing using Thermo Sequenase Terminator Cycle Sequencing Gel kit and [α-^{33}P]ddNTPs from Amersham. The corresponding encoded proteins were expressed in the TNT SP6-coupled transcription–translation system (Promega) but in an uncoupled way. Thus, transcriptions were carried out in 25-μl reactions containing 40 mM Tris–HCl (pH 8), 6 mM $MgCl_2$, 2 mM spermidine, 50 μg/ml BSA, 10 mM DTT, 1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP, 1 U/μl RNasin (Promega), 0.1 U/μl SP6 RNA polymerase (Promega) and 1 μg of linearized plasmid. After incubation at 37°C for 2.5 h, 10 U RNase-free DNase I (Promega) were added and incubated 15 min more at the same temperature. The RNA was extracted, precipitated, centrifuged and the pellet resuspended in 10 μl of TE (10 mM Tris–HCl (pH 8), 0.1 mM EDTA). Translations were performed following the manufacturer's protocol, but using 1 μl RNA instead of DNA and without SP6 RNA polymerase. When it was necessary to label proteins, [^{35}S]methionine (1000 Ci/mmol, Amersham) was used, and the incorporation of radioactive label calculated.

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Abbreviations: h-mtRPOL, human mitochondrial RNA polymerase; h-mtTFA, human mitochondrial transcription factor A; h-mtTFB, human mitochondrial transcription factor B; h-mTERF, human mitochondrial transcription termination factor

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2.2. RNA polymerase activity

Non-specific RNA polymerase activity was assayed with heat-denatured salmon sperm DNA as template as described before [13], with slight modifications. The reactions contained 10 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 10% (v/v) glycerol, 40 U RNasin, 50 μg/ml heat-denatured salmon sperm DNA (Sigma), 1 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP and 1μM [α-^{32}P]UTP (400 Ci/mmol, Amersham). Incubation was at 30°C for 30 min in presence of 10% (v/v) S-100 of a Tween-20 mitochondrial lysate [14] or 10% (v/v) of in vitro synthesized protein. Purification of synthesized RNA was carried out as described [14] and analyzed on a 5% (w/v) polyacrylamide gel containing 7 M urea. The gel was dried and exposed for autoradiography using an intensifying screen at −80°C. To determine the molecular activity of h-mtRPOLm, the transcription reactions were set up as described above. The reactions were terminated at different times by trichloroacetic acid precipitation and incorporated radioactive label was quantitated by scintillation counting [13]. The h-mtRPOLm molecular activity was calculated from the slopes of the curves that represents amount of UMP incorporated per amount of h-mtRPOLm versus time. Those values were corrected for reticulocyte lysate contribution. The molecular activity is defined as the number of molecules of UMP incorporated into acid-insoluble product in 1 min at 30°C per molecule of h-mtRPOLm.

Specific RNA polymerase activity was carried out under the same conditions as described above, but using *Eco*RI- and *Hin*dIII-digested pTER plasmid [5] at 20 μg/ml, as a template [14].

3. Results

3.1. In vitro expression of proteins known to be involved in human mitochondrial transcription

The cDNAs of the h-mtRPOL, h-mtTFA and h-mTERF subcloned in pSPUTK without the presequence were expressed in a rabbit reticulocyte lysate, to give mature proteins: h-mtRPOLm (1189 aa and 134578 MW), h-mtTFAm (204 aa and 24426 MW) and h-mTERFm (342 aa and 39187 MW). Fig. 1 shows that the gel mobility of the expressed ^{35}S-labeled proteins is in agreement with their theoretical molecular weight.

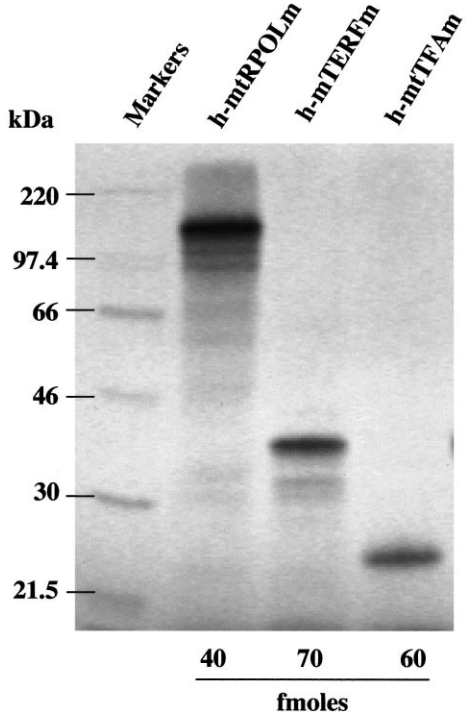

Fig. 1. SDS–PAGE analysis of the translation products synthesized in presence of [^{35}S]methionine.

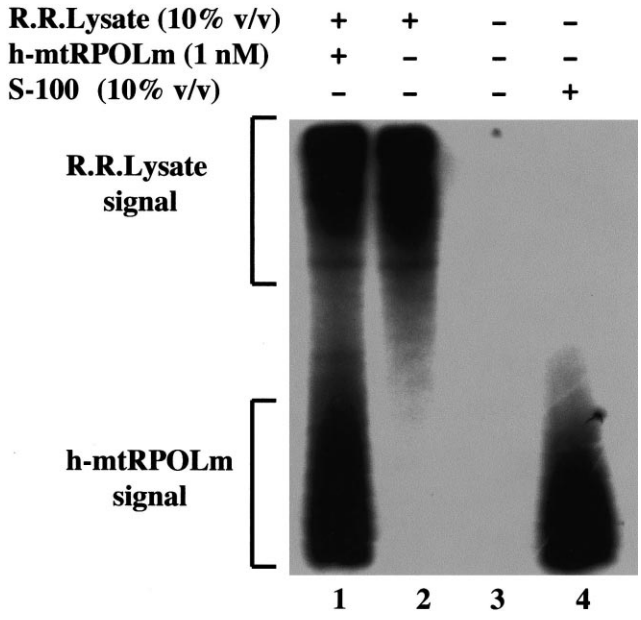

Fig. 2. Non-specific RNA polymerase activity. H-mtRPOLm (lane 1), S-100 lysate (lane 4) and two controls: rabbit reticulocyte lysate (lane 2) or no protein (lane 3).

3.2. Transcription activity assay

In order to determine if the recombinant h-mtRPOLm was active, the protein was assayed for RNA polymerase activity using a non-selective template (denatured salmon sperm DNA [13]) which does not require any additional factor. The h-mtRPOLm was expressed in vitro in a rabbit reticulocyte lysate system in an uncoupled way to avoid the presence of the SP6 RNA polymerase in the activity assay. The products from the activity assay were fractionated in a gel to discriminate the contribution of nuclear-contaminant RNA polymerase activity from the reticulocyte lysate. As shown in Fig. 2, h-mtRPOLm, at a concentration of 1 nM produced RNA synthesis of low and high molecular weight (lane 1). The synthesis of the high molecular weight RNA was also obtained when only reticulocyte lysate was tested (lane 2). This synthesis was probably due to the presence of some nuclear RNA polymerase in the reticulocyte lysate. On the other hand, when the transcriptional activity was assayed using human mitochondrial lysate S-100 [14], only RNA synthesis of low molecular weight was produced (lane 4). Therefore, the low molecular weight RNA obtained with h-mtRPOLm was due to its RNA polymerase activity, indicating that h-mtRPOLm is a true RNA polymerase. The molecular activity of h-mtRPOLm was calculated after correction for reticulocyte lysate contribution. A value of 2.1 ± 0.9 min^{-1} (average ± standard error of the mean of six independent experiments) was obtained.

The specific mitochondrial transcription activity was assayed using pTER template that contains the natural promoters and termination sequence of the human mtDNA [5] (Fig. 3). When a human mitochondrial S-100 was used, a clear RNA band from the L promoter was detected (lane 1), indicating that all the proteins necessary for initiating mitochondrial transcription were present. The use of reticulocyte lysate alone as a control to determine its interference in the assay did not show any signal (lane 2). Afterwards, h-mtRPOLm

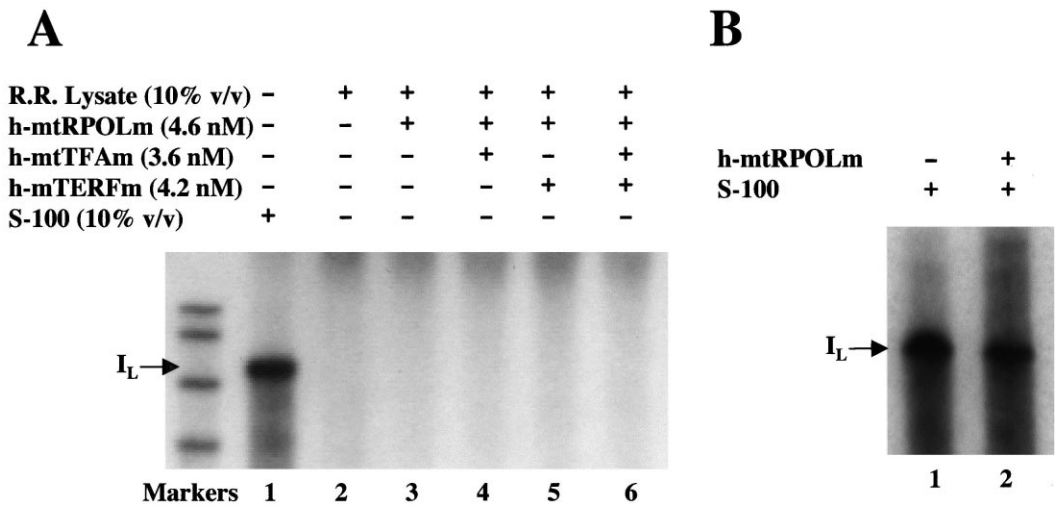

Fig. 3. Specific mitochondrial RNA polymerase activity. A: S-100 (lane 1), rabbit reticulocyte lysate (lane 2), h-mtRPOLm alone (lane 3) or in addition of h-mTERFm (lane 4) or h-mtTFAm (lane 5) or both together (lane 6). B: Transcription activity of S-100 in absence (lane 1) or presence (lane 2) of h-mtRPOLm. I_L, runoff transcripts from LSP; markers, pBR322 plasmid *Msp*I-digested.

was used in the specific transcription assay. Although h-mtRPOLm had RNA polymerase activity, by itself it was unable to initiate mitochondrial transcription from its natural promoters (lane 3). This result points to the necessity of other factor(s) to initiate mitochondrial transcription. The DNA binding activity of h-mtTFAm and h-mTERFm synthesized in vitro, to their target sequences was confirmed by mobility shift assay (data not shown). The addition of h-mtTFAm at a concentration that has been shown previously to activate transcription [15] (lane 4), h-mTERFm (lane 5) or both of them (lane 6) to h-mtRPOLm produced none effect in the initiation of the transcription. In conclusion, a factor(s) different to mtTFA and mTERF is required to promote specificity to the promoter.

When the recombinant h-mtRPOLm was added to the assay with the mitochondrial S-100 (Fig. 3B, lane 2), the transcription did not increase with respect to the mitochondrial lysate alone (lane 1), suggesting that h-mtRPOLm is not a limiting component of the transcription system.

3.3. *h-mtRPOL activity domains*

In order to gain insights into the role of different regions of h-mtRPOL in its RNA polymerase activity, a series of deletion mutants were derived from h-mtRPOLm (Fig. 4A). The mutants were expressed in vitro in an uncoupled way (Fig. 4B) and then assayed for their non-specific RNA polymerase activity (Fig. 4C). The recombinant h-mtRPOLm had higher activity that mitochondrial lysate S-100, while the mutants ΔN406, ΔC100 and ΔC400 had just residual activity and ΔNC2 and ΔNC3 had no activity, indicating that all regions deleted in the different mutants are essential for RNA polymerase activity.

4. Discussion

In the present work, the recombinant mature protein, h-mtRPOLm, was found to have non-specific RNA polymerase activity. Nevertheless, by itself it was not able to support specific transcription initiation from its natural human mitochondrial promoters. Addition of recombinant h-mtTFAm and/or h-mTERFm was not able either to produce any level of specific initiation activity. This observation indicates that another component(s) is necessary to confer this specific activity. The nature of this other component(s) is presently under investigation in our laboratory.

As concerns the structure of h-mtRPOLm, the central observation made in the present work is that the entire recombinant protein is required for the non-specific RNA polymerase activity.

The h-mtRPOL cDNA was identified by cyberscreening and the predicted protein has a high identity with the carboxy-terminal half of the sequence of the yeast mtRPOL, with a leader peptide that directs the protein into mitochondria [10]. But remaining to be proved is the principal function of this protein, its RNA polymerase activity. As shown in Fig. 2, the h-mtRPOLm synthesized in a mammalian system (rabbit reticulocyte lysate) has non-specific activity, concluding that h-mtRPOL is a true human mitochondrial RNA polymerase. A molecular activity of 2.1 ± 0.9 min^{-1} was calculated for h-mtRPOLm.

In the yeast *Saccharomyces cerevisiae*, mitochondrial transcription initiation requires two essential components: a non-specific sc-mtRPOL that retains the core catalytic activity [16], and a specificity factor required for promoter recognition, the sc-mtTFB [7]. In addition, a sc-mtTFA is required for the maintenance of mtDNA and stimulates 3–4-fold the transcription in vitro in the presence of sc-mtRPOL and sc-mtTFB, but is not able to substitute the sc-mtTFB protein [17]. As in yeast, transcription of the *Xenopus laevis* mitochondrial genome is performed also by two separate components [18]: the core xl-mtRPOL that exhibits non-specific transcription and a dissociatable factor required to confer specificity, the xl-mtTFB, that has been purified but has yet to be cloned. Furthermore, the xl-mtTFA, stimulates specific transcription in vitro by xl-mtRPOL and xl-mtTFB, but it is not required for basal transcription [8].

In contrast to the cases described above, human mitochondrial transcription initiation in vitro from mitochondrial promoters requires a mitochondrial RNA polymerase fraction, which has not been purified to homogeneity, and other tran-

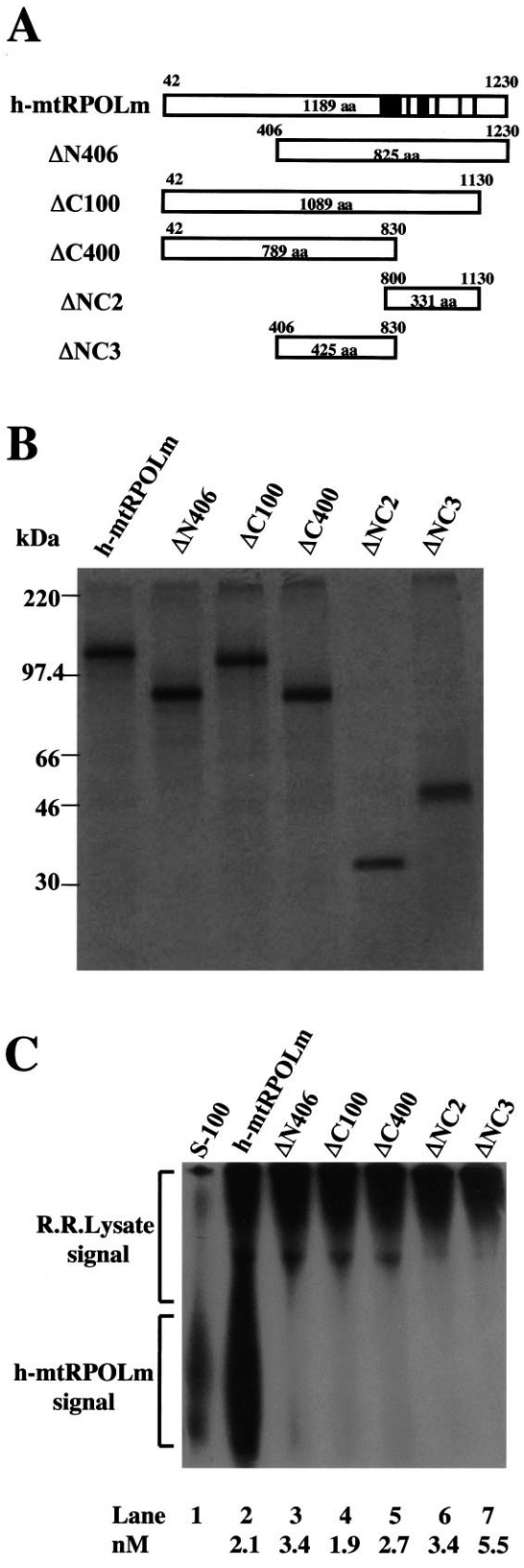

Fig. 4. Analysis of the h-mtRPOL mutants. A: Schematic representation of h-mtRPOLm and deleted constructs. B: SDS–PAGE analysis of the translation products. C: Non-specific RNA polymerase activity. The conserved peptide domains between h-mtRPOL and the T3 RNA polymerase are indicated by black boxes and the numbers represent the amino acid positions [10] in A. The positions of molecular mass markers are indicated in B.

scription factor, h-mtTFA, necessary for high levels of transcription [6]. The h-mtTFA has the ability to wrap and unwind DNA in vitro, in an essentially non-specific protein–DNA complex [19]. It has been pointed that h-mtTFA could be somehow able to provide the functions of both yeast transcription factors, sc-mtTFA and sc-mtTFB, due to h-mtTFA has an additional carboxy-terminal extension as well as a linker region between the two HMG box domains [19]. However, xl-mtTFA and h-mtTFA contain highly conserved sequences in these two regions that are not contained in yeast mtTFA [8] and in *X. laevis* also exists mtTFB, indicating that h-mtTFA may be not play the role of a mtTFB.

In this work it is presented an in vitro mitochondrial transcription assays where the h-mtRPOL is not from a purified fraction, but a recombinant protein. The expression of h-mtRPOLm in an in vitro mammalian system guarantees the presence of possible post-translational modifications in the recombinant protein, and the absence of any additional mitochondrial transcription factor in the assays. Under these conditions, h-mtRPOLm does not initiate transcription from human mitochondrial promoters when used in a concentration previously shown to have non-specific RNA polymerase activity, and that this activity is even higher than that produced by the S-100 (see Figs. 3 and 4C). This initiation was neither obtained when h-mtTFAm was added to the assay. It was used at a stoichiometry of one molecule of h-mtTFA per 13 000 bp of linear template DNA (3.6 nM). High levels of transcription have been observed when h-mtTFA was used at stoichiometry between 23 000 and 2300 bp per molecule (0.7–7 nM) [15], a range that includes the concentration used in this work. Under these conditions there is no specific transcription initiation, discarding the role of h-mtTFA as a component of the basal transcription machinery.

Up to now, only another transcription factor involved in human mitochondrial transcription has been cloned, the h-mTERF [12]. In order to check if h-mTERF can play some function in transcription initiation, the recombinant protein was added to the transcription assays. The h-mtRPOLm was unable to initiate transcription in presence of h-mTERFm even when h-mtTFAm was included in the mixture. Under this evidence the h-mTERFm is also discarded as a specificity factor.

The possibility that the absence of specific activity in the assays is due to the inhibition by rabbit reticulocyte lysate was discarded because S-100 was able to produce specific activity in its presence (Fig. 3B).

The data present in this work demonstrate that h-mtRPOL itself has no specificity for its natural promoters, while h-mtTFA and h-mTERF are not the specificity factor in human mitochondrial transcription system. So, it must exits other factor(s) that confers specificity to h-mtRPOL, that is, a human equivalent mitochondrial transcription factor B (h-mtTFB).

Finally, the analysis of the functional regions in h-mtRPOL in this work was unable to define any protein truncations that retained the RNA polymerase activity, indicating that multiple regions encompassing the entire length of the protein are involved in this activity.

Recently, the h-mtRPOL has been expressed in a non-mammalian expression system [20] and the purified protein was active in RNA synthesis from poly(dA-dT) template but not for the promoter-specific activity in presence of h-mtTFA.

These authors concluded that additional factor(s) may be needed, or that some mammalian-specific modification is missing in h-mtRPOL expressed in *Escherichia coli*. The results of the present paper point to the first possibility and discard the second one, since the h-mtRPOLm was expressed in a mammalian expression system.

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