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Transcription of mammalian mRNAs by a novel nuclear RNA polymerase of mitochondrial origin

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Abstract

It is commonly known that transcription of eukaryotic genes is carried out by three nuclear RNA polymerases of which RNA polymerase II is thought to be solely responsible for the synthesis of mRNAs¹. Here we show that transcription of some mRNAs in humans and rodents is mediated by a previously unknown single-polypeptide nuclear RNA polymerase (spRNAP-IV). The spRNAP-IV is expressed from an alternative transcript of the mitochondrial RNA polymerase (mtRNAP) gene (*POLRMT*). The RNAP IV^{SP} lacks 262 N-terminal amino acids of mtRNAP, including the mitochondrial-targeting signal, and localizes to the nucleus. Transcription by spRNAP-IV is resistant to the RNA-polymerase II inhibitor α -amanitin but sensitive to siRNA specific for the *POLRMT* gene. The promoters for spRNAP-IV differ substantially from those utilized by RNAP-II, do not respond to transcriptional enhancers, and contain a common functional sequence motif.

Replication and expression of the eukaryotic mitochondrial genomes fully depend on the nuclear genome: during evolution, most of the genes derived from the DNA of the proto-mitochondrial endosymbiont and required for the mitochondrial function were translocated to the nucleus such that most components of the mitochondrial proteome are imported into the mitochondria^{2,3}. The mitochondrial RNA polymerase (mtRNAP) is a homolog of bacteriophage single-polypeptide RNA polymerases, which displaced the typical, multisubunit bacterial RNA polymerase during the evolution of the endosymbiont^{4,5}, and is encoded in the spliced 3.8 kb transcript⁶ from the nuclear gene *POLRMT*. The human mtRNAP precursor has an N-terminal mitochondria-targeting peptide⁶, which is cleaved from the protein during mitochondrial import⁷. The N-terminal structure of mtRNAP is supported by 19 available Expressed Sequence Tags (ESTs); however, three ESTs correspond to an alternative structure, with a longer Exon 1 including the proximal 224 bp of Intron 1. The ORF encoded by the alternative transcript is truncated at the N-terminus and would specify a protein lacking the mitochondria-targeting peptide.

To verify the existence and to explore potential functions of an alternative, possibly non-mitochondrial product of the *POLRMT* gene, we designed primers to reveal variations within the 5' region of the *POLRMT* transcripts. By sequencing RT-PCR products produced with these primers, we found that a fraction of the transcripts in human, mouse and rat cell lines correspond to the splice variant retaining Intron 1 sequences (Supplementary Fig 1a,b), confirming that the transcripts specifying an N-terminally truncated mtRNAP are produced in both primates and rodents.

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To determine whether the shorter polypeptide lacking the mitochondrial targeting peptide is indeed synthesised, we analysed proteins that react with antibodies to a C-terminal peptide of mtRNAP⁸. Confocal microscopy revealed both mitochondrial and nuclear localisation of immunoreactive proteins in HeLa cells (Fig. 1a). On Western blots, total cell extracts showed two protein bands of ~135kD and ~110 kD. In isolated mitochondria, there was a single band of ~135kD, whereas nuclear extracts contained only the ~110kD band (Fig 1b). Total extracts from the mitochondrial DNA-depleted p0 cells⁹ (Supplementary Fig 2a) contained reduced amounts of the mitochondrial 135 kD form, whereas the nuclear 110 kD protein was unaffected (Fig 1b). Inhibition of *POLRMT* gene by RNAi resulted in abrogation of both nuclear and mitochondrial immunostaining (Supplementary Fig 2b) and almost complete disappearance of the two protein forms (Fig. 1b) and both transcripts from *POLRMT* gene (Supplementary Fig. 1a). By contrast, RNAi against the proximal portion of Intron 1 caused abrogation of nuclear immunostaining concomitant with the disappearance of the nuclear 110 kD band, with no changes in mitochondria (Fig 1d; Supplementary Fig 2b).

Over-expression of the cDNA encoding the full-length mtRNAP⁶ caused a substantial increase in the mitochondrial 135kD band but no increase in the 110kD nuclear form (Fig 1c, Supplementary Fig 2b), suggesting that the latter protein does not originate from the mtRNAP mRNA, be it via internal initiation of translation or via processing of the 135kD precursor. We then over-expressed in p0 HeLa cells the predicted alternative ORF and, separately, the mtRNAP ORF, both tagged with a C-terminal flag epitope. Immunostaining revealed exclusively mitochondrial localization of the over-expressed mtRNAP and exclusively nuclear localization of the protein encoded in the alternative ORF (Fig 2a). The electrophoretic mobility of the over-expressed flag-tagged proteins corresponded precisely to the two endogenous bands revealed with *POLRMT*-specific antibodies (Fig 2b), although the size of the nuclear protein determined by electrophoresis (~110 kD) was much smaller than the size of the alternative ORF product predicted from the mRNA sequence (~130 kD). Attaching an N-terminal flag to the alternative ORF resulted in a protein that did not react with anti-flag antibodies but reacted with antibodies to mtRNAP (Fig 2c), suggesting that the first ATG was not used for the translation initiation. Indeed, the product of an N-terminally truncated alternative ORF fragment starting at the first initiation codon conserved between mouse and human (ATG6) was identical in size to the endogenous nuclear protein whereas the fragment starting at ATG8 produced a smaller protein (Supplemental Figs 3 and 4). Thus, the nuclear form of the protein appears to start at ATG6 of the ORF and correspond to the 968 C-terminal amino acids of mtRNAP, which include the entire catalytic domain (Supplementary Fig 3).

Our findings suggest that the *POLRMT* gene specifies two single-polypeptide polymerases, one (mtRNAP) targeted to the mitochondria, and the other (spRNAP-IV) having a putative function in the nucleus. Conceivably, spRNAP-IV could be the fourth nuclear RNA polymerase of animal cells, although alternative functions of this protein, other than direct involvement in transcription, could not be ruled out. To determine whether or not spRNAP-IV is important for cell viability, we inhibited the expression of *POLRMT* gene by siRNA expression. This resulted in a substantial growth retardation and cell death which could be reversed by co-expression of recombinant spRNAP-IV (Fig. 2d). Thus, spRNAP-IV seems to play a distinct role in cell physiology.

In an attempt to identify genes that could be transcribed by the putative spRNAP-IV, we hypothesized that transcription of these genes would be resistant to α -amanitin, a specific inhibitor of RNAP-II. Hybridization of a total RNA probe from HeLa cells treated with α -amanitin with an Affymetrix microarray U133A revealed ~70 up-regulated (twofold or greater) transcripts. Of the 15 transcripts tested by RT-PCR, four showed lack of sensitivity to or even stimulation by α -amanitin (Supplementary Fig 5 a,b). A similar pattern was observed for the orthologous transcripts in mouse 3T3 cells (Supplementary Fig 5c). In Northern hybridisation,

the α -amanitin-resistant transcripts encoding the Zn-finger BTB domain-containing protein 1 (*ZBTB1*), prenylcysteine oxidase (*MGC3265*), and aldehyde dehydrogenase 8A1 (*ALDH12*) genes showed dose-dependent stimulation by α -amanitin, whereas control transcripts from the *p21^{WAF1/CIP1}* (*CDKN1A*) and *GAPDH* genes were sensitive to α -amanitin (Fig 3a; Supplementary Fig 6). When RNAP-I or RNAP-II was inhibited by RNAi, gradual decrease and disappearance of transcripts encoding each of these polymerases was observed by day 4, which correlated with substantial stimulation of *ZBTB1*, *MGC3265* and *ALDH12* transcripts (Fig 3b). Inhibition of the RNAP-III largest subunit with siRNA oligonucleotides also resulted in slightly increased levels of all three transcripts (Supplemental Fig. 6b). Thus, these genes apparently are not transcribed by known nuclear RNA polymerases. By contrast, inhibition of *POLRMT* transcripts in p0 HeLa cells by RNAi was accompanied by a dramatic decrease in transcripts from the α -amanitin-resistant genes (Fig 3c), which was reversed by over-expression of the spRNAP-IV ORF (Fig 3d). These results indicate that the truncated nuclear protein product of the *POLRMT* gene indeed participates in the production of certain mRNAs.

To examine the repertoire of transcripts affected by spRNAP-IV, we monitored changes in the expression pattern in p0 HeLa cells following 48 hours expression of siRNA to *POLRMT* gene using hybridization to Affymetrix cDNA U133 2.0 plus microarray. Of ~20,000 transcripts that gave a positive signal, ~1,000 transcripts were substantially inhibited (two-fold or greater), while ~300 transcripts showed comparable levels of up-regulation (Supplementary Table). Certainly, some of the changes could be attributed to secondary effects of inhibition of the true spRNAP-IV-regulated genes. Also, cooperation between spRNAP-IV and RNAP-II in the expression of certain genes cannot be ruled out. Of 17 randomly selected transcripts that showed down-regulation on the microarray, 9 demonstrated an α -amanitin-resistant/inducible expression when assayed by RT-PCR (not-shown), suggesting that mammalian spRNAP-IV-dependent genes might count in hundreds. Up-regulation of some of these transcripts by α -amanitin was confirmed by Northern analysis in three different p0 cell lines (Supplementary Fig. 7).

To analyse promoters used for initiation of transcription by spRNAP-IV, we amplified and cloned into a reporter plasmid the upstream regions of *ALDH12* and *ZBTB1* genes. When transfected into HeLa cells, both putative spRNAP-IV promoters supported luciferase expression, with activities comparable to that of the SV40 early promoter (Fig. 4a). The expression of luciferase driven by the *ALDH12* and *ZBTB1* promoters was resistant to α -amanitin, in contrast to the α -amanitin-sensitive expression from the SV40 promoter (Fig. 4a). Inhibition of *POLRMT* by RNAi 24 hrs before transfection of reporter constructs led to a decreased activity of the *ALDH12* promoter but did not affect the SV40 promoter (Fig. 4b). To determine whether spRNAP-IV binds to the *ALDH12* promoter, formaldehyde-fixed extracts of HeLa cells transfected with an empty vector or with the constructs bearing *ALDH12* and SV40 promoters were precipitated with antibodies to RNAP-II or to *POLRMT*, and the number of ampicillin-resistant colonies indicating the efficiency of immunoprecipitation was determined by transformation of *E. coli*. For the *ALDH12*-promoter-containing plasmid, substantial enrichment was observed with the *POLRMT* antibodies; a 10-fold lower enrichment was seen with antibodies to RNAP-II suggesting that spRNAP-IV preferentially binds to this construct. The reciprocal enrichment was observed with the SV40 promoter construct (Fig 4c).

A comparison of the upstream sequences of the *ALDH12*, *ZBTB1*, and *MGC3265* genes revealed two shared motifs (Supplementary Fig 8); counterparts to these motifs were identified also in several additional genes putatively transcribed by spRNAP-IV, including *ULK1* (data not shown). Both motifs were conserved among the orthologous genes from other mammals (Supplementary Fig 8). The putative spRNAP-IV promoter motifs showed no detectable similarity to the sequences of RNAP I-III promoters^{10,11}, those of the known transcription-

factor-binding sites¹², the sequences of mtDNA that are involved in mtRNAP-directed transcription or the promoters of T-odd bacteriophage genes transcribed by single-polypeptide phage polymerases homologous to mtRNAP. Deletion of the sequences spanning these motifs from the *ALDH12*, *ZBTB1* and *MGC3265* promoters abrogated α -amanitin-resistant expression of luciferase, suggesting that the motifs are, indeed, functional elements of spRNAP-IV promoters (Supplementary Fig 9).

Transcription initiated by RNAP-II can be substantially activated by transcription factors which bind to transcriptional enhancers¹. We tested whether the activity of promoters of spRNAP-IV-controlled genes could be stimulated by placing a strong cytomegalovirus (CMV) enhancer close to *ALDH12*, SV40 and mitochondrial DNA H-strand (mtHs) promoters in a luciferase reporter plasmid. Upon transfection into HeLa cells, the CMV enhancer stimulated α -amanitin-sensitive transcription from the SV40 promoter more than 10-fold. By contrast, the *ALDH12* promoter fragment did not respond to the enhancer at all, and its basal activity remained responsive to stimulation by α -amanitin. Similarly, in HeLa cells producing the artificial transcription factor tTA¹³, the activity of *ALDH12* promoter did not change when coupled with the tetracycline-responsive element (Supplementary Fig 10), confirming that RNAP-II enhancers do not affect transcription by spRNAP-IV. The mtHs promoter was inactive in this system, suggesting that the regulation of spRNAP-IV transcription in the nucleus is distinct from that of the mtRNAP in the mitochondria (Fig. 4c).

Identification of the spRNAP-IV as a second polymerase transcribing mRNAs in mammals raises a wealth of questions awaiting future investigation. In particular, it remains to be determined at what stage of eukaryotic evolution was a single-polypeptide RNA polymerase recruited for a nuclear role, what is the repertoire of genes transcribed by spRNAP-IV, how is the spRNAP-IV-dependent transcription regulated, how RNAP-II and spRNAP-IV divide and/or share their tasks in the transcription of mRNAs, and whether spRNAP-IV participates in gene silencing, similarly to the recently identified structurally-unrelated RNAP IV of *Arabidopsis*^{14,15}. A further important question is what are the transcription factors that affect spRNAP-IV functions. The facts that spRNAP-IV promoters share no sequence similarity with mtDNA and that three known transcription factors (mtTFA, mtTFB1 and mtTFB2) which cooperate with mtRNAP seem to localize exclusively to the mitochondria^{16–18} suggest that spRNAP-IV might utilise a distinct set of factors for transcription of nuclear genes.

Methods

Cell culture

Human carcinoma cell lines HeLa, A431, H1299 and RKO, normal human lung fibroblasts MRC5, mouse fibroblasts 3T3 and rat fibroblasts Rat1 were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and penicillin/streptomycin. Mitochondrial DNA-deficient p0 HeLa were obtained, subcloned and cultured as described¹⁹. The absence of mtDNA was confirmed by PCR. The mitochondria in control and p0 cells were stained with MitoTracker Red 589 (Invitrogen, Carlsbad, CA).

Northern analysis

Radioactive probes were prepared from DNA inserts of appropriate I.M.A.G.E. Consortium clones: *RNAP I large subunit* – I.M.A.G.E. 4413694; *RNAP II large subunit* – I.M.A.G.E. 4359716; *POLRMT* – I.M.A.G.E. 6572256; *GAPDH* – MGC:2031, I.M.A.G.E. 3543589; *p21 (CDKN1A)* – MGC:97077, I.M.A.G.E.7262289; *ALDH12* – I.M.A.G.E. 4998434; *MGC3265* – I.M.A.G.E. 3506623; *ZBTB1* – MGC:60335, I.M.A.G.E. 6141266.

RT-PCR amplification

Total RNA isolated by Trizol reagent was converted to cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR amplifications (typically 20–25 cycles) were carried out with primers specified in figure legends.

Recombinant constructs

Inhibition of expression of different genes was achieved by infection with recombinant lentiviral vector constructs **pLSLG** expressing hairpin siRNAs controlled by H1 RNA gene promoter as previously described²⁰. The following 19 – 21 bp regions corresponding to appropriate mRNAs were present in the hairpin transcripts: *RNAP I large subunit*: 5'-gtttacaagccactgaatcgc and 5'-gaaaccagcttcagtttctg; *RNAP II large subunit*: 5'-gaactattctccaaccagtc and 5'-gatacaccacagcttccaa; Exon 3 of *POLRMT* gene 5'-caaagatactggagaaggata; Exon 17 of *POLRMT* gene - 5'-caacacagtaagcagaagaa; Intron 1 of *POLRMT* gene 5'-ggcaaagaaggaacacaa. For expression of recombinant mtRNAP cDNA⁶ (gift from Dr. Valeria Tiranti, National Neurological Institute, Italy) or truncated forms of mtRNAP we used modified lentiviral expression vector **pLV**²¹ (from Dr. Inder Verma, Salk Institute, La Jolla, CA). Preparation of high-titer lentiviral stocks and infection of target cell cultures was as described²¹.

Chromatin immunoprecipitation

pGL3-luc (Promega Inc.) constructs containing 278 bp upstream region of human *ALDH12* gene (–290 – –12) or 202 bp SV40 early promoter were lipofected into HeLa cells. The cells were fixed with 1% formaldehyde 24 hours thereafter, the prepared cell extracts³⁰ were incubated overnight with either antibodies to mtRNAP (1:200) or antibodies to RNAP II (N-2, sc-899, Santa Cruz Biotech, CA) and Protein G Agarose beads). The beads were washed six times with 20mM Tris-HCl, 200mM NaCl, 0.5% Triton-X100, 0.05% deoxycholate, 0.1% NP-40, 1mM PMSF, containing 250mM LiCl, and precipitates were eluted with 1% SDS and 200mM NaCl. The samples were heated at 65°C for 6 hours, extracted with phenol/chloroform, ethanol-precipitated and transfected as five independent parallel samples into competent *DH5α E.coli*. The number of ampicillin-resistant colonies representing immunoprecipitated plasmids was counted.

Microarray hybridisations with total RNA from HeLa or p0 HeLa cells treated with either 10 µg/ml α-amanitin for 12 hours, or 48 h after introduction of siRNA-expressing lentivirus vector, were carried out using U133A or U133 2.0 Plus human cDNA microarrays at Gene Expression Array Core Facility (Case Western Reserve University, Cleveland, Ohio) according to Affymetrix protocols.

Western analysis

Samples of total protein, mitochondrial and nuclear fractions were isolated as described²², heated at 95°C, separated in 4–12% SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies raised to a peptide (VNLEPSDVPQDVY) of human mtRNAP (gift of Gerald Shadel, Emory University School of Medicine, Atlanta, GA) or anti-FLAG antibodies M2 (Sigma-Aldrich, St.Louis, MO). The filters were developed with peroxidase-labeled anti-rabbit polyclonal antibodies and ECL-Plus Western detection reagents (Amersham Biosciences, Piscataway, NJ).

Immunostaining

Cells were fixed with 4% formaldehyde in PBS for 20 min, washed and incubated overnight at 4°C with anti-mtRNAP or anti-Flag M2 antibodies (1:200) in blocking buffer²². TRITC-

labelled anti-rabbit, or FITC-labelled anti-mouse (FITC) antibodies (Jackson Immune Research) were used for immunostaining.

Luciferase assays for promoter activity

Upstream regions of *ALDH12*, *ZBTB1*, *MGC3265* genes and mitochondrial DNA H-strand promoter (mtHs) were cloned into **pGL3-luc** reporter vector (Promega US, Madison, WI). The plasmids were transfected into HeLa or p0 HeLa cells using Lipofectamine reagent (Invitrogen, Carlsbad, CA), along with a plasmid expressing β -galactosidase served for normalisation. The activity of luciferase was measured in cell extracts with Luciferase Assay System (Promega US, Madison, WI.) using fluorimetric plate reader. .

Computational methods

Amino acid sequence alignments were constructed using the MACAW program²³. The search for nucleotide motifs in promoter regions and statistical assessment of the detected motifs was performed using the ParaMEME²⁴, Gibbs sampling²⁵, CONSENSUS²⁶, and MULTIPROFILER²⁷ methods. Search for known transcription-factor-binding sites was performed using the MATCH program²⁸. The conservation of the promoter sequences in orthologous mammalian genes was determined using the BLAT server²⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Confocal microscopy of HeLa cells stained with antibodies to *POLRMT* peptide and TRITC anti-rabbit IgG counterstained for nuclei with Hoechst 33258. (B) Two *POLRMT* gene products detected by Western in HeLa (three left lanes) and $\rho 0$ HeLa cells (two right lanes) with antibodies to *POLRMT* peptide. Total-cell extracts - lanes 1,4,5; mitochondrial (lane 2) and nuclear (lane 3) fractions; sample from cells expressing siRNA to Exons 3 and 17 of human *POLRMT* gene (lane 5); (C) Expression of mtRNAP-encoding ORF in $\rho 0$ HeLa cells (Western); (D) Expression of siRNA to *POLRMT* Intron 1 in $\rho 0$ HeLa cells (Western with antibodies to *POLRMT* peptide).

Figure 2.

Two distinct *POLRMT*-specific polypeptides are encoded by alternative transcripts. (A) Immunostaining with anti-Flag antibodies of HeLa cells transduced with flag-tagged ORF for mtRNAP (left) and with flag-tagged alternative ORF encoding spRNAP-IV (right); (B) Western analysis of proteins reactive with antibodies to a *POLRMT* peptide following overexpression in total cell extracts of mtRNAP ORF (lanes 2 and 4), or spRNAP-IV ORF (lanes 3 and 5) in total-cell extracts (lanes 2 and 3) or in the nuclei (lanes 4 and 5); (C) Western analysis of HeLa cells transduced with spRNAP-IV Flag-tagged at the N- or C-terminus and developed with antibodies to *POLRMT* (left) or to Flag (M2). (D) Expression of *POLRMT* siRNA affects cell growth. Specified p0 cell lines were infected with lentivirus vector **LSLG** co-expressing GFP and siRNA to *POLRMT* Exon 3 (or to Exon 17, which produced similar result) or GFP and siRNA to luciferase, mixed with uninfected cells, and the proportion of fluorescing cells was monitored by FACS. A control sample of H1299 cells was co-infected with lentivirus vector expressing spRNAP-IV ORF (day 14 point marked by an arrow).

Figure 3.

(A) Dose-dependent stimulation of *ZBTB1*, *MGC3265* and *ALDH12* transcripts in HeLa and MRC5 cells treated for 14 hours with α -amanitin (Northern); (B) Time-dependent stimulation of the transcripts following infection of HeLa cells with lentiviral vector **LSLG** expressing siRNAs to RNAP I or RNAP II. Inhibition of RNAP I and II mRNAs is shown on lower panels (Northern); (C) Inhibition of the transcripts four days after infection of p0 HeLa cells with LSLG lentivirus expressing siRNA to *POLRMT* (Northern), and (D) stimulation of the same transcripts after infection with a lentiviral vector expressing spRNAP-IV ORF (RT-PCR).

Figure 4.

(A) Transcription from the *ALDH12* and *ZBTB1* promoters depends on spRNAP-IV. **pGL3-luc** constructs bearing *ALDH12* (−482 −12) and *ZBTB1* (−330 -- +12) promoters were transfected into HeLa cells (48 hours). The cultures were treated with α -amanitin for 12hrs (luciferase assay); (B) Suppression of *ALDH12* promoter by siRNA to *POLRMT*. 24hrs after infection of HeLa cells with siRNA expressing **LSLG** constructs the cells were transfected with **pGL3-ALDH12** (−482 −12 upstream fragment) or control plasmids and assayed for luciferase in 48 hrs; (C) Chromatin immunoprecipitation with antibodies to RNAP II or *POLRMT* of promoter-containing plasmids transfected into HeLa cells. The plasmids contained either *ALDH12* (−290 −12) or SV40-promoters. (D) CMV-enhancer (−600 −112)

does not cooperate with ALDH12 promoter (−290 −12). 48 hours after transfection of promoter-bearing **pGL3-luc** constructs (ALDH12 −290 − −12, mtH-strand promoter, SV40 early promoter) the cultures were treated with α -amanitin for 12hrs and assayed for luciferase.