

Human RNase P ribonucleoprotein is required for formation of initiation complexes of RNA polymerase III

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Received May 15, 2014; Revised April 24, 2015; Accepted April 24, 2015

ABSTRACT

Human RNase P is implicated in transcription of small non-coding RNA genes by RNA polymerase III (Pol III), but the precise role of this ribonucleoprotein therein remains unknown. We here show that targeted destruction of HeLa nuclear RNase P inhibits transcription of 5S rRNA genes in whole cell extracts, if this precedes the stage of initiation complex formation. Biochemical purification analyses further reveal that this ribonucleoprotein is recruited to 5S rRNA genes as a part of proficient initiation complexes and the activity persists at reinitiation. Knockdown of RNase P abolishes the assembly of initiation complexes by preventing the formation of the initiation sub-complex of Pol III. Our results demonstrate that the structural intactness, but not the endoribonucleolytic activity per se, of RNase P is critical for the function of Pol III in cells and in extracts.

INTRODUCTION

RNA polymerase III (Pol III) transcribes a large number of small non-coding RNA genes, including the 5S rRNA, tRNA and U6 snRNA genes (1–4). Pol III consists of numerous protein subunits, some of which are specific for this enzyme, while others are shared with Pol I and Pol II (1–4). The specific subunits RPC3, RPC6 and RPC7 form a dissociable initiation subcomplex required for the polymerase to carry out promoter-dependent initiation (5–7). The transcription cycle of Pol III consists of initiation, elongation, termination and reinitiation (2,8–10). Pol III needs core transcription factors and accessory proteins for accurate and efficient initiation (2,4,11–14). In vitro transcription studies reveal that various combinations of the core transcription factors TFIIIA, TFIIIB, TFIIIC and SNAPc form distinct preinitiation complexes that recruit Pol III to different target genes (2,15). Formation of initiation com-

plexes is relatively fast and followed by start of RNA polymerization (16–18). TFIIIA, TFIIIC and SNAPc function as assembly factors, whereas TFIIIB facilitates the correct positioning of the polymerase at the start point (19,20). Pol III requires TFIIIB for reinitiation on tRNA and SNR6 genes, and TFIIIB and TFIIIC for reinitiation on longer gene templates, such as the yeast SCR1 gene (11). Nonetheless, Pol III can catalyze the first round of initiation at a 3' overhanged linear DNA template (G-less tDNA¹⁶) without transcription factors, but it requires TFIIIB and TFIIIC for reinitiation (21).

We have previously shown that human RNase P has a role in transcription of small non-coding RNA genes by Pol III in cells and in extracts (22–25). Human nuclear RNase P is an endoribonuclease that processes the 5' leader sequence of precursor tRNA (ptRNA) and it has at least 10 distinct protein subunits associated with an RNA species, termed H1 RNA (26–28). The protein subunits are designated Rpp14, Rpp20, Rpp21, Rpp25, Rpp29, Rpp30, Rpp38, Rpp40, Pop1 and Pop5 (27–30). Targeted destruction of H1 RNA or its cognate protein subunits, including Rpp21, Rpp29 and Rpp38, abolishes transcription of 5S rRNA, tRNA, U6 snRNA and 7SL RNA genes, an indication that these subunits act in the context of RNase P (22,24). Nuclear run-on assays demonstrate that RNase P is critical for the nascent transcription of 5S rRNA genes (22,24). Moreover, chromatin immunoprecipitation analyses reveal that protein subunits of this ribonucleoprotein (RNP) bind to active 5S rRNA and tRNA genes (22,24). However, the molecular mechanism by which RNase P exerts its effect on transcription remains unknown.

In this study, we show that HeLa nuclear RNase P is a part of proficient initiation complexes recruited to 5S rRNA gene, and that this RNP is critical for the assembly of these complexes in cells and in extracts.

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MATERIALS AND METHODS

Recruitment of initiation complexes to biotinylated 5S rRNA gene templates

For affinity purification of initiation complexes of Pol III, three biotinylated DNA fragments containing a cloned human 5S rRNA gene (22) were generated by PCR using 5' biotinylated deoxyoligonucleotides (see Supplementary Table S1). These fragments were 333, 629 and 1003 bp in length. As controls, biotinylated DNA fragments that corresponded to the human RNU1-1 gene, which codes for U1 snRNA, and the multiple cloning site of pBluescript (SK) (Supplementary Table S1) were produced. Each DNA fragment (100 ng) was immobilized to streptavidin beads (1 μ l), which were first washed three times with 1 \times binding buffer (8 mM Tris-HCl, pH 7.5, 0.5 mM EDTA and 1 M NaCl) in total volumes of 200 μ l for overnight in cold room. The immobilized DNA was washed three times with 1 \times wash buffer (15 mM Tris-HCl, pH 7.5, 80 mM KCl, 5 mM MgCl₂, 0.3 mM DTT, 0.12 mM EDTA, 20 mM creatinine phosphate and 12% (v/v) glycerol). The DNA was then added to HeLa whole cell extracts (S20) diluted in 1 \times assembly buffer (12 mM Tris-HCl, pH 7.9, 80 mM KCl, 5 mM MgCl₂, 0.5 mM DTT and 20 mM sodium creatine phosphate) in the presence or absence of 0.5 mM of rNTP. Each assembly reaction contained 90 μ l of extract (protein concentration was 10–15 mg/ml) and 10 μ l of beads coupled to DNA. After 1 h of incubation on ice, the beads were separated by a magnet, washed four times with 1 \times assembly buffer, resuspended in 100 μ l of the same buffer and then were transferred to new tubes. Complexes bound to the DNA were subjected to western blot analysis, RNase P and Pol III assays, and RT-PCR.

For RT-PCR of H1 RNA, the primer H1-RP1 or H1-RP2 (Supplementary Table S1) was utilized for separate reverse transcription of H1 RNA. The resulted cDNAs were amplified by Pfu DNA polymerase using the forward primer H1-FP1 (Supplementary Table S1) and reverse primer H1-RP1 or H1-RP2, thus generating PCR products of 340 and 100 bp in length, respectively.

Velocity sedimentation analysis in glycerol gradients

Assembly of initiation complexes on a human 5S rRNA gene was carried out after modification of a method initially designed for formation of initiation and elongation complexes of Pol II (31). Thus, 200 μ l of HeLa whole cell extracts were mixed with 1.25 μ g of a circular or Afl III-digested pBluescript(SK) containing a genomic human 5S rRNA gene in 1 \times assembly buffer [10 mM Tris-HCl, pH 7.9 at 4°C, 8 mM MgCl₂, 20 mM (NH₄)₂SO₄, 5 mM sodium creatine phosphate, 2 mM DTT and 0.5 mM ATP]. The 0.4-ml mixture was then incubated for 10 min at 30°C to allow for formation of initiation complexes before it was layered on a 4.4-ml volume glycerol gradient [15–40% v/v glycerol, 20 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 40 mM (NH₄)₂SO₄, 0.2 mM EDTA, pH 8.0 and 2 mM DTT] prepared in 12 \times 51 mm polyallomer tubes. Centrifugation was performed at 250 000 g (50 000 rpm) for 180 min at 4°C in an ultracentrifuge using TST55.5 rotor. Fractions of 0.2 ml in volume were collected from the top of the gradient.

Aliquots of 50 μ l from each fraction were immediately assayed for Pol III activity in transcription reactions of 55 μ l in volume in the presence of 0.5 mM of rNTP and 10 μ Ci of [α -³²P]UTP. In parallel, fractions were assayed for RNase P activity in processing of the 5' leader sequence of an internally ³²P-labeled *S. pombe* ptRNA^{Ser} (pSupS1) or *E. coli* ptRNA^{Tyr} in 1 \times MRP/TNET buffer containing 0.3 units of rRNasin, 150 ng of cold ptRNA and 10 mM DTT, as previously described (27). Quantitation of the values of the cleavage products (bands) was done by the use of the EZQuant-Gel software for densitometric quantitation.

For fine resolution of initiation complexes, the assembly procedure described above was carried out in a large-scale mixture of 0.8 ml in volume. The mixture was overlaid on a 11.2-ml glycerol gradient (5–30%) in 14 \times 89 mm polyallomer tubes. Centrifugation was performed for 6 h at 126 000 g (32 000 rpm) in SW41 Ti rotor. Fractions of 0.25 ml in volume were collected and aliquots of 50 μ l from each fraction were immediately assayed for Pol III and RNase P, as detailed above.

For velocity sedimentation analysis of reinitiation complexes, the above procedure was repeated, but the assembly mixture was further incubated for 30 min at 30°C in the presence of rNTP (0.5 mM each) to enable ongoing transcription by conversion of committed initiation complexes to reinitiation ones.

Gel filtration analysis of initiation complexes of Pol III

Initiation complexes were assembled on a human 5S rRNA gene, as described above. The assembly mixture (0.8 ml) was then loaded onto a 100-ml Sephacryl S-500 HR column, which was prewashed with 10 volumes of 1 \times transcription buffer [20 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 40 mM (NH₄)₂SO₄, 0.2 mM EDTA, pH 8.0, 2 mM DTT and 20% v/v glycerol]. This column is designed to separate macromolecules in the molecular mass range of 4×10^4 – 2×10^7 Da (GE Healthcare). Fractions of 2.5 ml were collected and aliquots of 0.1 ml from each fraction were assayed for transcription by addition of rNTPs (0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.05 mM UTP) and 10 μ Ci of [α -³²P]UTP. Fractions were also examined for the activity of RNase P in processing of ptRNAs and for the presence of its protein subunits by western blot analysis using specific antibodies.

In Supplementary Figure S3A, initiation complexes were fractionated on a Sephacryl S-400 HR column (60-ml volume), which is designed to resolve macromolecules in the molecular mass range of 2×10^4 – 8×10^6 Da (GE Healthcare), and then the most proficient complexes were further purified by velocity sedimentation analysis using a 15–40% glycerol gradient. The activities of Pol III and RNase P in the resulted fractions were determined, as described above.

Cell transfection, *in vitro* transcription, chromatin immunoprecipitation and RNase H digestion assays

See (22,24) for specific details.

RESULTS

Targeted cleavage of H1 RNA inhibits 5S rRNA gene transcription at initiation

H1 RNA in HeLa whole cell extracts (S20) was targeted for cleavage by the use of recombinant RNase H and antisense H1-1 deoxyoligonucleotide (Figure 1B and Supplementary Figure S1A), which corresponded to the specificity domain of H1 RNA (Figure 1A), as previously described (22). Cleavage of H1 RNA resulted in aberrant processing of ptRNA^{Tyr} (Figure 1C, lane 4 versus 2; bracket) and led to inhibition of transcription of a human 5S rRNA gene, when added to the pretreated extract (Figure 1D, lane 2 versus 1; Figure 1E). This aberrant processing of ptRNA^{Tyr} was manifested in inhibition of the correct cleavage of the 5' leader sequence by RNase P, possibly due to the confined excision of the specificity domain of H1 RNA shown to mediate substrate recognition (30), and enhanced degradation of the 5' leader sequence in ptRNA^{Tyr}, thus generating mature-like tRNA. By contrast, the use of a scrambled or sense oligonucleotide did not reveal comparable effects on processing of ptRNA^{Tyr} (Figure 1C, lanes 3 and 5) or transcription (Figure 1D, lanes 1 and 3).

To check if H1 RNA is implicated in initiation, the 5S rRNA gene was added to an extract to elicit the formation of initiation complexes, before H1 RNA was targeted for cleavage, as specified above. Remarkably, transcription occurred in this post-treated extract (Figure 1D, lane 5 versus 2), even though RNase P was efficiently targeted, as manifested in the anomalous processing of ptRNA^{Tyr} (Figure 1C, lane 7 versus 2). By contrast, the control oligonucleotides mediated minor effects on tRNA processing (Figure 1C, lanes 6 and 8) and transcription (Figure 1D, lanes 4 and 6).

The results demonstrate that H1 RNA is involved in initiation of 5S rRNA gene transcription, apparently prior to the formation of initiation complexes (see below).

RNase P is recruited to 5S rRNA genes

To check if H1 RNA is recruited to the 5S rRNA gene, a biotinylated DNA fragment containing the 5S rRNA gene was utilized for affinity purification of initiation complexes from extracts (see Materials and Methods section)(10). This short DNA fragment (333 bp in length) was found to elicit the assembly of initiation complexes, which were transcriptionally efficient as those assembled on larger DNA fragments with extended sequences flanking the 5S rRNA gene (Figure 2A, lane 7 versus 8 and 9, and lanes 4–6).

Purified initiation complexes assembled on the short biotinylated DNA fragment contained the full length H1 RNA subunit of RNase P, as determined by RT-PCR analysis (Figure 2B, lanes 3 and 11). The two bands that flanked the H1 RNA-derived PCR product were non-specific ones (Figure 2B, lane 5 and 13; asterisks). The signal of the specific product increased when the assembly of initiation complexes was performed in extracts preincubated with rNTPs (Figure 2B, lanes 2 and 10 versus 3 and 11), probably due to the addition of ATP, shown to promote the formation of initiation complexes (31). Additionally, purified initiation complexes contained protein subunits (Figure 2C, two

upper panels) and the activity (Supplementary Figure S1B, lanes 4–6 and 7–9 versus 13–15 and S1C; 5' band) of RNase P. By contrast, the activity of this endonuclease was barely pulled down by the use of the control DNA fragment (Supplementary Figure S1B, lanes 13–15; MCS). A biotinylated DNA fragment that harbored the RNU1-1 gene, which codes for U1 snRNA, brought down little RNase P activity (Supplementary Figure S1B, lanes 10–12 versus 13–15, and S1C), but the significance of this reproducible outcome needs further examination, which will take into consideration that the U1 snRNA genes are transcribed by Pol II (32).

The results show that a fraction of nuclear RNase P in HeLa whole cell extracts is recruited to the 5S rRNA gene, possibly as an integral part of initiation complexes (see below).

RNase P copurifies with proficient initiation complexes

Initiation complexes assembled on the 5S rRNA gene carried on plasmid were subjected to velocity sedimentation analysis using a 15–40% glycerol gradient (see Materials and Methods section)(31). Fractions collected from the gradient were assayed for transcription and processing of ptRNA^{Ser}. Transcription of the 5S rRNA gene occurred in fraction F8, peaked at fraction F10, and then gradually decreased toward the bottom of the gradient (Figure 3A). This transcriptional activity coincided with that of RNase P (Figure 3B and C), which existed in large heterogeneous complexes with molecular weights of 1×10^6 to 3×10^6 , as estimated from the molecular weights of the 40S small ribosomal subunit ($\sim 1.5 \times 10^6$) and 80S ribosomes ($\sim 4 \times 10^6$; Figure 3E, F10–F18). For comparison, a partially purified form of HeLa RNase P (27) is settled at fraction F6 of equivalent glycerol gradients (Supplementary Figure S2E). The activities of Pol III and RNase P were not abundant in the upper fractions, i.e. F2–F4, which were rather enriched with Rpp20, Rpp25, Rpp40 and RPC7 (Figure 3D), an indication that these proteins were also present in smaller sub-complexes.

Remarkably, the contribution of the plasmid DNA, which harbored the 5S rRNA gene, to the molecular weights of the initiation complexes found in fractions F10–F18 was insignificant, as inferred from the low sedimentation velocity of the plasmid alone (Figure 3F; F6). Hence, substantial protein content should have contributed to the high molecular weights of these initiation complexes. Of note, there was no extensive overlap between the plasmid carrying the 5S rRNA gene and Pol III activity, particularly in F14–F16 (Figure 3A versus E), even though transcription was dependent on the presence of this gene (Supplementary Figure S2A). Therefore, this plasmid seems to elicit the assembly of non-specific DNA-protein complexes in extracts.

To verify that the above transcription was carried out by de novo assembled initiation complexes (Figure 3A), a control extract without 5S rRNA gene was subjected to velocity sedimentation analysis. As expected, the resulted fractions did not synthesize 5S rRNA (Supplementary Figure S2A). However, when the transcription reactions were repeated in the presence of 5S rRNA gene, fraction F8 exhibited a moderate Pol III activity (Supplementary Figure S2B, lanes 4–

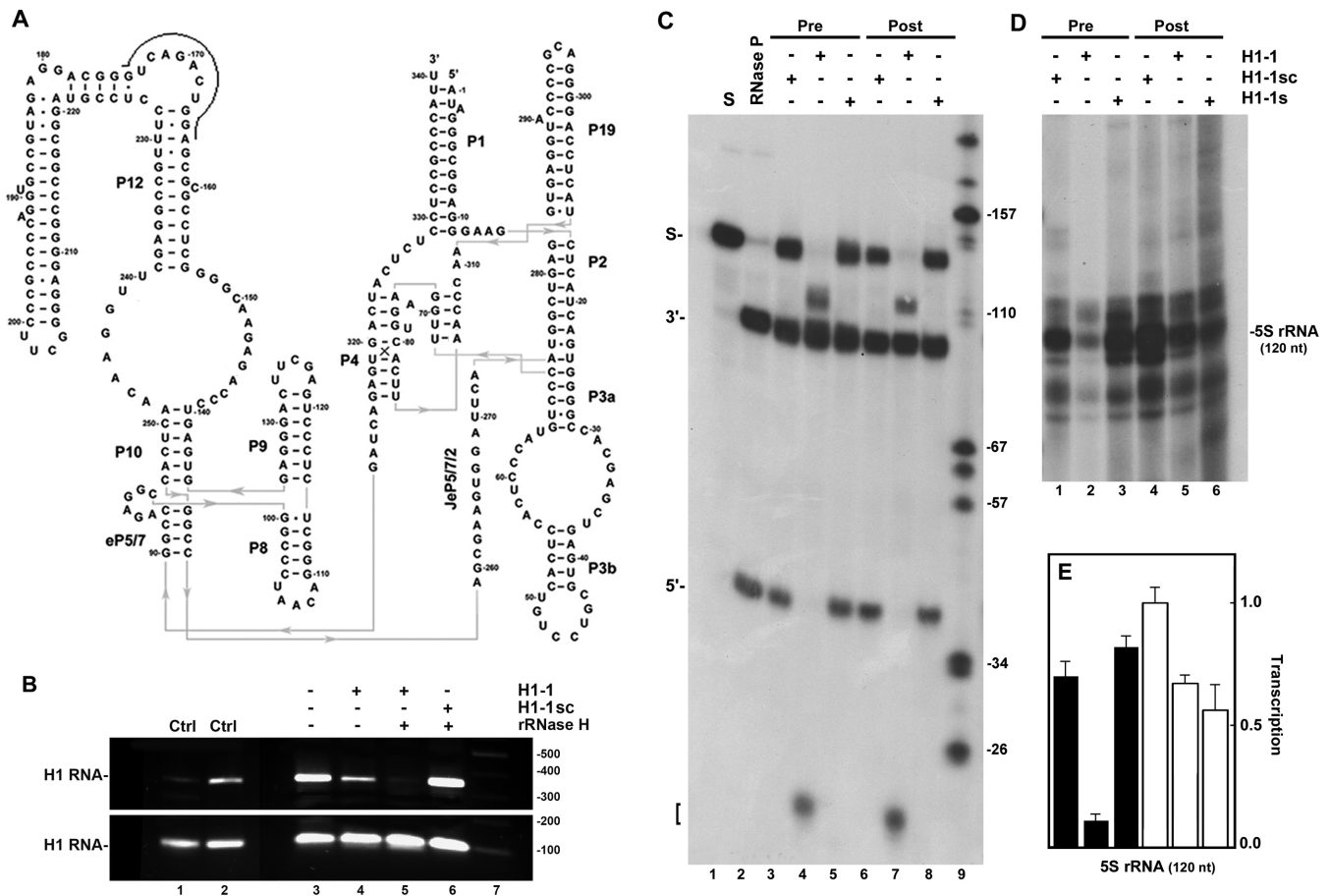


Figure 1. Targeted cleavage of H1 RNA inhibits 5S rRNA gene transcription, if cleavage precedes initiation complex formation. (A) A predicted secondary structure of H1 RNA. The antisense H1-1 oligonucleotide is shown. (B) Upper panel: RT-PCR analysis of the full length H1 RNA in extracts subjected to RNase H digestion assay using recombinant RNase H and H1-1 (lane 5 versus 3) or H1-1sc (lane 6 versus 3) oligonucleotide (22). The assay was performed for 50 min at 30°C. Cleavage of H1 RNA seen in the presence of the H1-1 oligonucleotide alone (lane 4 versus 3) was mediated by endogenous RNase H (see Supplementary Figure S1A). Lower panel: RT-PCR analysis using a reverse primer positioned upstream of the RNase H cleavage site in H1 RNA (see Materials and Methods section). A product of 100 bp in length was produced in all extracts (lanes 3–6). Lanes 1 and 2 represent positive controls for PCR using genomic DNA and for RT-PCR using *in vitro* transcribed H1 RNA, respectively. Lane 7 depicts DNA ladders. (C) Extracts were pretreated with RNase H in the presence of the H1-1, H1-1sc or H1-1s oligonucleotide (lanes 3–5) or they were initially incubated with the 5S rRNA gene for assembly of initiation complexes and then H1 RNA was targeted for cleavage (lanes 6–8). All extracts were tested for processing of the 5' leader sequence of pTRNA^{Tyr} by RNase P for 20 min. Aberrant processing of the 5' leader sequence (bracket) and generation of shortened pTRNA^{Tyr} (band between S and 3') were seen (lanes 4 and 6). A DEAE-purified HeLa RNase P was used as a control for normal processing of pTRNA^{Tyr} (lane 2). The positions of the substrate (S), tRNA (3') and 5' leader sequence (5') are indicated. A DNA size marker is shown in lane 9. (D) Transcription of the 5S rRNA gene was tested in the pretreated (lanes 1–3) and post-treated (lanes 4–6) extracts described in (A). The position of 5S rRNA is indicated. (E) Relative 5S rRNA gene transcription seen in (D). Bars represent densitometric quantitation of the 5S rRNA bands. Error bars are shown for three independent experiments.

6). This result indicates that the core transcription factors essential for transcription of the 5S rRNA gene existed in fraction F8, which was highly enriched with active RNase P (Supplementary Figure S2B versus C).

Comparison of the sedimentation velocities of RNase P and Pol III in the control extract described above with those of the two enzymes in initiation complexes revealed a marked shift of the latter complexes toward the downstream fractions, i.e. F10–F24 (Supplementary Figure S2D versus Figure 3C). These fractions corresponded to molecular weights equivalent to those of the 40S and 80S ribosomal particles (Figure 3E). Hence, RNase P and Pol III were recruited to the 5S rRNA gene as components of massive initiation complexes. Nevertheless, the extent of the recruitment of these two enzymes to initiation complexes was

limited (see Supplementary Figure S2B, lane 5, versus Figure 3A, lane 6), possibly due to the experimental constraint of having an adjusted DNA (gene): protein ratio in the extract, in order to obtain maximal transcription output.

RNase P persists in initiation and reinitiation complexes

To corroborate that the initiation complexes assembled on the 5S rRNA gene incorporate RNase P, these large heterogeneous complexes were resolved by gel filtration chromatography using a Sephacryl S-500 HR column (see Materials and Methods section). Competent initiation complexes were excluded from this column in numerous fractions, i.e. F29–F41, but the peak of the transcription activity was evident in F33 (Figure 4A). The molecular weights of these initiation complexes ranged from 1×10^6 to 3×10^6 , as as-

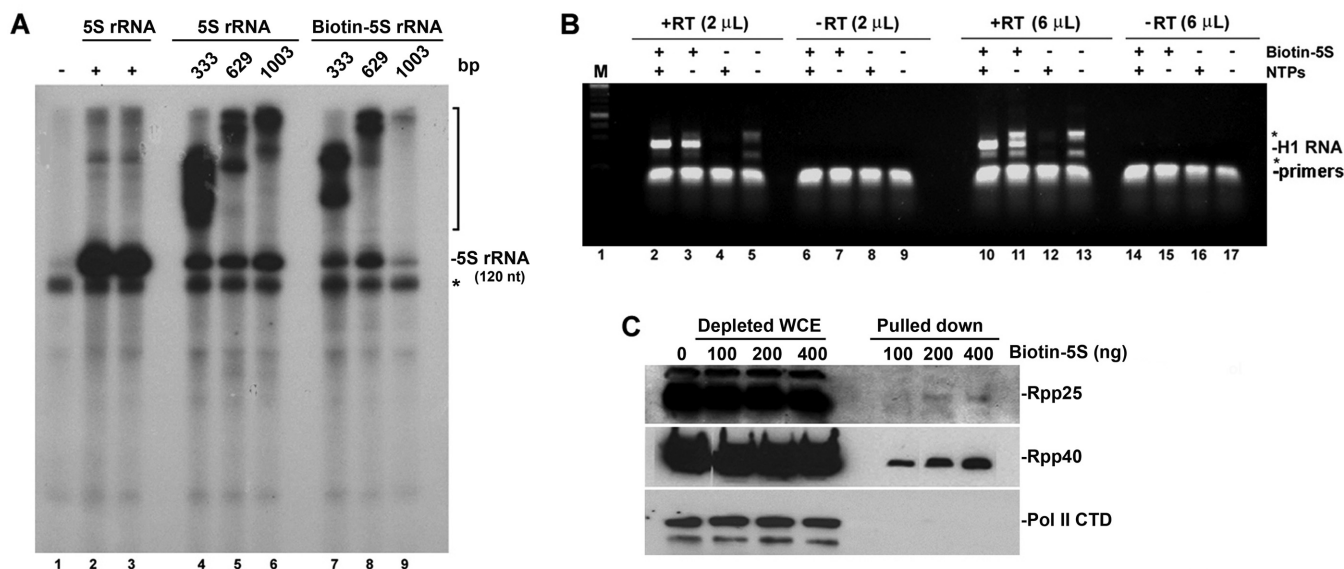


Figure 2. RNase P is recruited to 5S rRNA genes. (A) Transcription of 5S rRNA genes in non-biotinylated (lanes 4–6) and biotinylated (lanes 7–9) DNA fragments of the indicated sizes was carried out in HeLa whole cell extracts (see Materials and Methods section). The position of 5S rRNA is shown. Bracket indicates elongated transcripts, probably generated by an inaccurate initiation or termination of transcription (lanes 2 and 3). Asterisk points to an internally labeled non-specific RNA (lane 1). (B) RT-PCR analysis of H1 RNA in initiation complexes formed on biotinylated (lanes 2, 3, 6, 7, 10, 11, 14 and 15) or non-biotinylated (lanes 4, 5, 8, 9, 12, 13, 16 and 17) DNA fragments harboring the 5S rRNA gene. These fragments, 333 bp in length, were incubated in extracts in the presence or absence of rNTPs. Asterisks point to non-specific bands (see lanes 5 and 13). Aliquots of 2 and 6 μ l from the RT reactions were used for PCR to check for the reaction specificity (e.g. lane 2 versus 10). PCR reactions without RT were included as negative controls (lanes 6–9 and 14–17). A DNA size marker is shown in lane 1. (C) Purified complexes were subjected to western blot analysis of Rpp25, Rpp40 and the CTD of RPB1 of Pol II. Left lane represents the protein signals in the control extract.

sessed from the molecular weights of the 40S and 80S ribosomal units (Figure 4A; arrowheads). Likewise, RNase P was detected in fractions F31–F41, but its maximal activity was seen in F33 (Figure 4B and C). By contrast, Pol II was excluded later from the column, as revealed by the presence of the distinctive carboxy terminal domain of its RPC1 subunit in fractions F37–F39 (Figure 4B), whereas microtubule polymers were identified already in F20 (Figure 4B, lower panel).

The persistent copurification of RNase P with Pol III was also evident when initiation complexes purified by gel filtration chromatography were further analyzed by velocity sedimentation using a glycerol gradient (Supplementary Figure S3A; F14–F22). Moreover, these enzymes remained together when initiation complexes committed to the 5S rRNA gene were converted to reinitiation complexes under conditions of ongoing transcription (Supplementary Figure S3B; see Materials and Methods section).

Disruption of RNase P prevents the formation of proficient initiation complexes

RNase P was targeted in HeLa cells by the use of a siRNA directed against its protein subunit Rpp25, which belongs to the Alba-like superfamily of chromatin binding proteins (33). Knockdown of Rpp25 showed no effect on the expression of other protein subunits, except for that of its interacting partner Rpp20 (Supplementary Figure S4A, lanes 1–3 versus 4–7), which is known to be subject to coordinate inhibition (24), but led to severe inhibition of 5S rRNA gene

transcription in extracts derived from the siRNA-treated cells (Supplementary Figure S4B and C). Surprisingly, these extracts showed normal RNase P activity in processing of tRNA^{Ser} (Supplementary Figure S4D) or tRNA^{Tyr} (data not shown). These opposite effects on transcription and tRNA processing were prominent in 48 h of transfection of cells, in which the steady state level of Rpp25 decreased by \sim 90% (Supplementary Figure S4A, lane 2 versus 5), the transcription capability of Pol III declined by \sim 92% (Supplementary Figure S4B, lane 3 versus 6, and C), whereas the activity of RNase P remained largely unaffected (Supplementary Figure S4D). Transcription was very low in extracts prepared from siRNA- or mock-treated cells after 66 h of transfection (Supplementary Figure S4B, lanes 4 and 7, and C), probably due to the cessation of proliferation of the confluent cells (22). The inhibition of transcription of the 5S rRNA gene in extracts derived from cells deficient in Rpp25 was partially rescued by the addition of a highly purified recombinant Rpp25 protein (Supplementary Figure S4E), an indication that the deficiency in this protein did not eliminate the biosynthesis of other components essential for transcription (see below).

Velocity sedimentation analysis revealed that the formation of initiation complexes on the 5S rRNA gene in extracts deficient in Rpp25 was defective, when compared with that of the control extracts (Figure 5B, F12–30, lower versus upper panel). This defect was associated with marked retardations in the sedimentation patterns of residual Rpp25, RPC6 (Figure 5C versus D) and RNase P (Supplementary Figure S5A versus B; F6–F12). Similarly, a striking shift

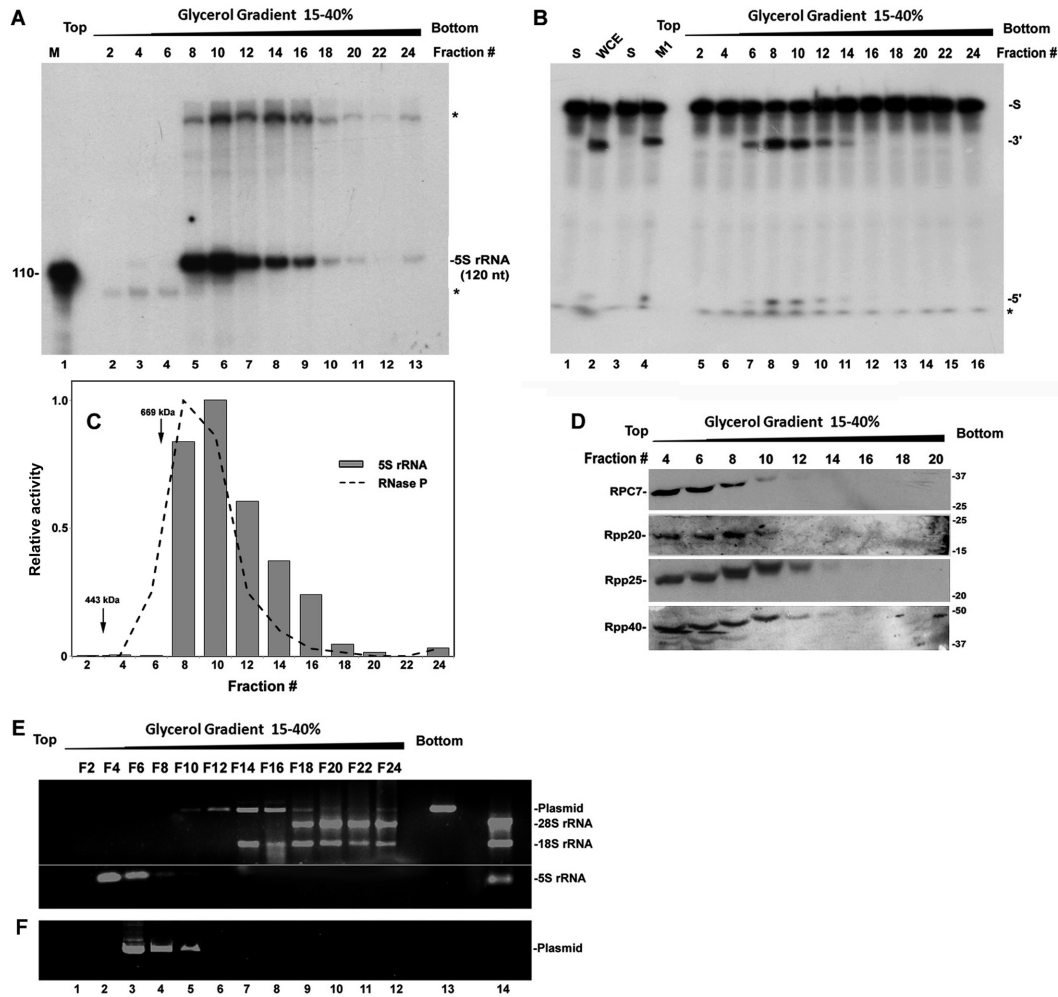


Figure 3. Velocity sedimentation analysis of initiation complexes assembled on a human 5S rRNA gene. (A) Velocity sedimentation analysis in a 15–40% glycerol gradient of initiation complexes assembled on the 5S rRNA gene in HeLa whole cell extract. Fractions were collected from the top of the gradient and were assayed for transcription of the 5S rRNA gene. The position of the 5S rRNA is indicated. Upper asterisk depicts large transcripts, which were possibly generated by inaccurate initiation and/or termination of the polymerase. Lane 1 shows a 110-nt RNA size marker. The gel was exposed to autoradiography for 12 h. (B) Processing of ptRNA^{Ser} by RNase P in fractions seen in (A). Degradation of the substrate is marked by an asterisk. (C) Relative activities of Pol III (bars) and RNase P (dashed line) in fractions seen in (A) and (B). The 5S rRNA band seen in (A) and the 5' leader sequence of ptRNA^{Ser} shown in (B) were quantitated and plotted. The positions of the protein weight standards, Thyroglobulin (669 kDa) and Apoferritin (443 kDa), which were analyzed in a parallel gradient, are shown. (D) Western blot analysis of Rpp20, Rpp25, Rpp40 and RPC7. Protein size markers (in kDa) are depicted. (E) Agarose gel electrophoresis of 5S rRNA (lower panel), 18S rRNA, 28S rRNA and the Afl III-digested plasmid carrying the 5S rRNA gene (top panel) after extraction from F2–24 seen in (A). The two panels represent the same gel. Each panel is a composite of two minigels. Lanes 13 and 14 show size markers of the digested plasmid and total RNA extracted from HeLa cells, respectively. (F) Velocity sedimentation analysis of an Afl III-digested plasmid carrying the 5S rRNA gene. The plasmid was visualized as described in E.

in the sedimentation velocity of the plasmid carrying the 5S rRNA gene was evident (Figure 5E versus F, F1–F10). Thus, these results demonstrate that the observed inhibition of 5S rRNA gene transcription in extracts deficient in Rpp25 was due to failure in the assembly of initiation complexes (see below).

Chromatin immunoprecipitation analysis confirmed that knockdown of Rpp25 (Figure 6A) prevented the binding of RPC6 to 5S rRNA genes in HeLa cells (Figure 6B, lane 1 versus 7 and 4 versus 10, respectively; Figure 6D, lane 8 versus 17). By contrast, the occupancy of these genetic loci by RPB6, RPB8 and RPC7 remained unaffected (Figure 6B, lanes 2, 3 and 5 versus 8, 9 and 11; Figure 6D,

Lanes 6 and 7 versus 15 and 16). Similarly, knockdown of Rpp25 prevented the binding of RPC6 to tRNA^{Tyr} genes (Figure 6E, lane 5 versus 11), and reduced that of RPC7 (Figure 6E, lane 4 versus 10), even though the steady state levels of these two proteins remained essentially unchanged (Figure 6C and A). This lack of chromatin occupancy by RPC6 and RPC7, which are components of the dissociable initiation subcomplex of Pol III (6,18,34,35), points to the recruitment of structurally defective initiation complexes to 5S rRNA and tRNA genes. This mechanistic explanation is supported by the marked decrease in the steady state level of 5S rRNA in cells lacking Rpp25 (Figure 5F, F8–F18, lower panel versus upper panel).

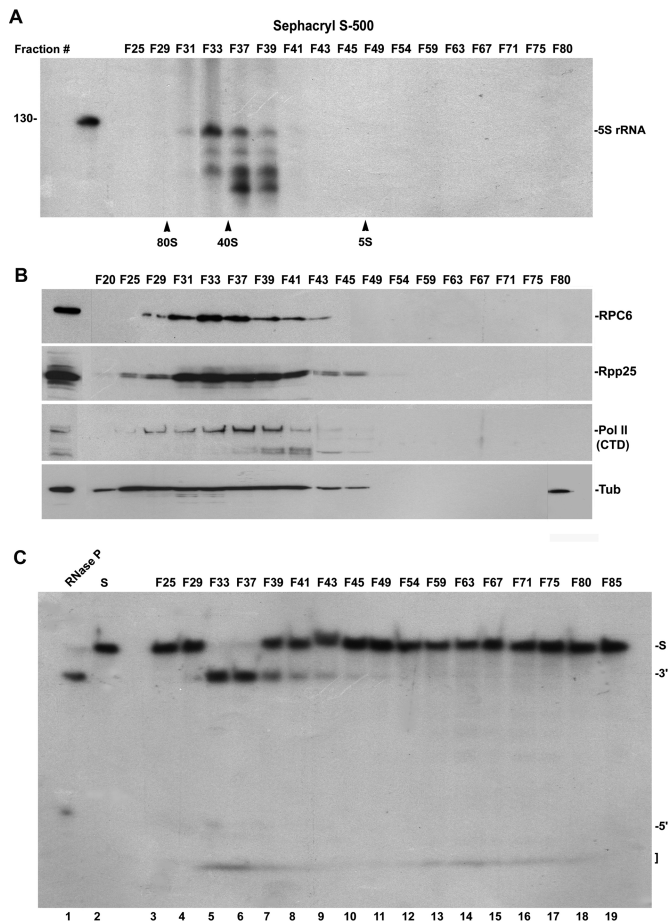


Figure 4. Gel filtration analysis of initiation complexes using a Sephacryl S-500 column. (A) Fractionation of initiation complexes assembled de novo on the 5S rRNA gene in HeLa whole cell extracts by a Sephacryl S-500 HR column. Eluted fractions were examined for the presence of proficient initiation complexes, as described in Figure 3A. Leftmost lane shows a 130-nt RNA size marker. The presence of the 5S, 40S and 80S particles (arrowheads) was determined as specified in Figure 3E. (B) Western blot analysis of RPC6, Rpp25, CTD of RPB1 of Pol II and α -Tubulin in fractions seen in (A). The signal of α -Tubulin in F80 represents a low molecular weight form. The leftmost lanes represent the load. This panel is composed of three blots. (C) Fractions seen in (A) were tested for the cleavage of pTrnA^{Ser} by RNase P. Bracket points to RNA degradation.

DISCUSSION

We have shown that HeLa nuclear RNase P is integrated into proficient initiation complexes assembled on the 5S rRNA gene, and that this RNP is required for the formation of these complexes in cells and in whole cell extracts, which are biologically relevant transcription systems. Moreover, these large heterogeneous initiation complexes do not require RNase P as a tRNA enzyme (30,36), but apparently as a scaffold for their proper assembly on 5S rRNA genes, thus revealing a new structural role for this RNP in the formation of type I transcription apparatuses.

Biochemical purification analyses demonstrate that an active form of HeLa nuclear RNase P copurifies with large preexisting complexes capable of transcribing 5S rRNA genes (Supplementary Figure S2B and D). These preexisting complexes have molecular masses of 1–2 million dal-

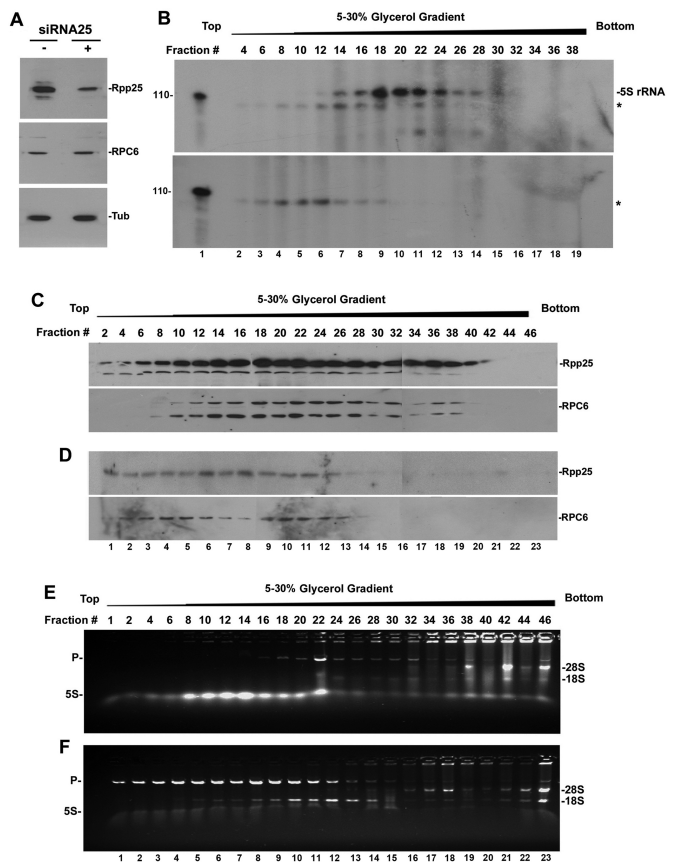


Figure 5. Knockdown of Rpp25 prevents formation of initiation complexes. (A) Western blot analysis of Rpp25, RPC6 and α -Tubulin in HeLa cells transfected for 48 h with a minigene expressing a siRNA directed against Rpp25 or with an empty plasmid (24). (B) Transcription of 5S rRNA gene by initiation complexes purified by velocity sedimentation in large-scale 5–30% glycerol gradients (see Materials and Methods section). Initiation complexes were derived from whole extracts of mock-transfected cells (with plasmid; upper panel) or cells transfected with a minigene expressing a siRNA against Rpp25 (lower panel). The leftmost lane shows a 110-nt RNA size marker. Asterisks point to labeled non-specific RNAs. (C and D) Western blot analysis of Rpp25 and RPC6 in fractions described in (B). Based on the exposure times of the blots, the level of Rpp25 in panel (D) was reduced by ~90%, when compared with that seen in (C). (E and F) Detection of 5S rRNA, 18S rRNA and 28S rRNA in the indicated fractions described in (B) was performed as in Figure 3E. The level of 5S rRNA was low (lower panel) due to inhibition of transcription of endogenous 5S rRNA genes in transfected cells deficient in Rpp25.

tons, and therefore they are much larger than the molecular masses of individual TFIIIA and TFIIIB (35), as well as RNase P (~460 kDa; Supplementary Figure S2D)(27). Though the subunit makeup of these preexisting complexes requires further analyses, TFIIIA should be part of these specialized complexes. The occurrence of preexisting Pol III holoenzymes dedicated to transcription of VA1 RNA and tRNA genes has been reported by other groups (see (35)). Moreover, our biochemical purification analyses show that proficient initiation complexes assembled de novo on 5S rRNA genes integrate RNase P. The estimated molecular masses of these latter complexes range from 1.5 to 3 million daltons. Hence, all the core and auxiliary transcription factors of the type I genes should have been co-assembled with Pol III and RNase P to generate these massive complexes.

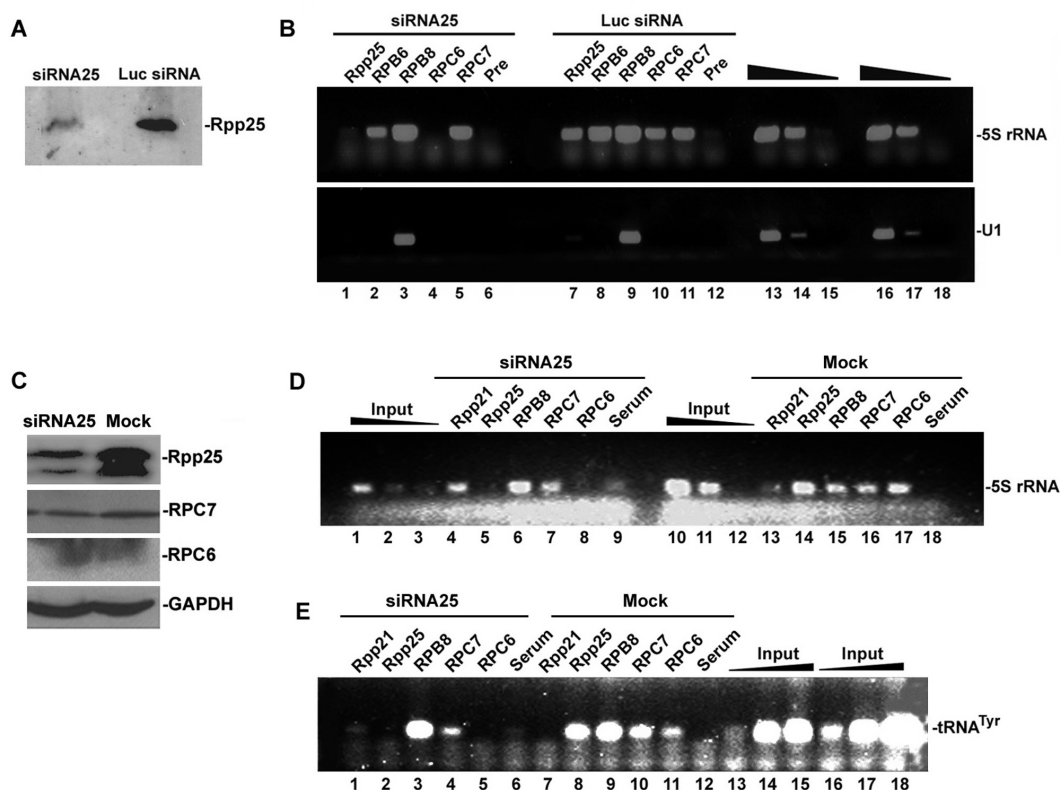


Figure 6. Lack of binding of specific subunits of Pol III to 5S rRNA and tRNA genes in cells deficient in Rpp25. (A) Western blot analysis of Rpp25 in HeLa cells transfected for 48 h with a siRNA directed against Rpp25, as in Figure 5A, or with a control luciferase siRNA. (B) Chromatin immunoprecipitation analysis of 5S rRNA and U1 snRNA genes in cells shown in (A) by the use of antibodies against Rpp25 (lanes 1 and 7), RPB6 (lanes 2 and 8), RPB8 (lanes 3 and 9), RPC6 (lanes 4 and 10), RPC7 (lanes 5 and 11) and preimmune rabbit serum (lanes 6 and 12). For linear PCR, 1, 0.1 and 0.01% of input DNA from cells transfected with an Rpp25 siRNA (lanes 13–15) or luciferase siRNA (lanes 16–18) are shown. (C) Western blot analysis of Rpp25, RPC7, RPC6 and GAPDH in cells transfected with Rpp25 siRNA or mock-transfected with an empty vector. (D and E) Chromatin immunoprecipitation analysis of 5S rRNA and tRNA^{Tyr} genes in the cells described in (C). The signal of binding of Rpp21 to tRNA^{Tyr} genes is typically weak.

HeLa nuclear RNase P fulfills all criteria to be considered a bona fide transcription factor for Pol III. First, this RNP is implicated in transcription of the three major types of genes transcribed by this polymerase and is critical for the activity of a type III promoter adjoined to a heterologous bacterial gene expressed in human cells (22,24). Second, this RNP is recruited to 5S rRNA genes and its activity persists at reinitiation. Chromatin immunoprecipitation experiments confirm that many RNase P protein subunits occupy transcriptionally active 5S rRNA genes in a cell-cycle-dependent manner (24; this study). Third, RNase P is vital for the recruitment of RPC6 to 5S rRNA genes, and thereby for the formation of the initiation subcomplex of Pol III. The observation that the recruitment of RPB6 and RPB8 to 5S rRNA and tRNA genes takes place without the enrollment of RPC6 and RPC7 demonstrates that the former subunits, which are shared with Pol I and Pol II, bind to these genes independently of the latter specific subunits. This finding also suggests that the formation of initiation complexes on 5S rRNA genes in cells might be different from that in extracts. Fourth, nascent 5S rRNA transcripts do not undergo endonucleolytic cleavages, a fact that opposes the prospect that RNase P is integrated into type I

transcription machineries as an adjunct endoribonuclease. In this regard, Pol III possesses an intrinsic RNA cleavage activity, but this asset rather complements its principal task as an RNA polymerase (37).

Finally, it has been shown that transcription of a human U6 snRNA gene could be reconstituted by the use of recombinant core transcription factors and purified Pol III (38). Apparently, RNase P is not required for transcription in this experimental system in which naked DNA is accessible to transcription factors. Nonetheless, our biochemical and functional analyses show that this RNP is critical for transcription of the U6 snRNA, 7SL RNA, tRNA and 5S rRNA genes in cells and in HeLa whole cell extracts (22,24; this study). Thus, it is possible that RNase P facilitates the recruitment of initiation complexes to 5S rRNA genes that exist in chromatin forms. If this possibility is correct, then the Alba-like chromatin binding protein subunit Rpp25, and its interacting partner Rpp20 that exhibits ATPase activity (39), may relate RNase P to active chromatin states of 5S rRNA genes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENT

We thank Prof. Peter Cook (University of Oxford, UK) for providing us with hybridomas and monoclonal antibodies directed against protein subunits of Pol III. R.S. and N.O. are grateful for doctoral fellowship support from the Hoffman Leadership and Responsibility Program and the Rudin Foundation, respectively.

FUNDING

United States-Israel Binational Science Foundation [2005/009 and 2011/052 to N.J. and S.A.]; Israel Science Foundation [368/11 to N.J.]. Funding for open access source: United States-Israel Binational Science Foundation [2011/052].

Conflict of interest statement. None declared.

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