A role for the catalytic ribonucleoprotein RNase P in RNA polymerase III transcription

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The physical and functional links between transcription and processing machines of tRNA in the cell remain essentially unknown. We show here that whole HeLa extracts depleted of ribonuclease P (RNase P), a tRNA-processing ribonucleoprotein, exhibit a severe deficiency in RNA polymerase (Pol) III transcription of tRNA and other small, noncoding RNA genes. However, transcription can be restored by the addition of a purified holoenzyme. Targeted cleavage of the H1 RNA moiety of RNase P alters enzyme specificity and diminishes Pol III transcription. Moreover, inactivation of RNase P by targeting its protein subunits for destruction using small interfering RNAs inhibits Pol III function and Pol III-directed promoter activity in the cell. RNase P exerts its role in transcription through association with Pol III and chromatin of active tRNA and 5S rRNA genes. The results demonstrate a role for RNase P in Pol III transcription and suggest that transcription and early processing of tRNA may be coordinated.

Keywords: RNase P; RNA polymerase III; small, noncoding RNA gene; catalytic ribonucleoprotein; tRNA processing

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Transfer of genetic information requires tRNA, which serves as the adaptor between mRNA and protein. This fundamental role of tRNA requires that newly transcribed primary transcripts of tRNA be accurately processed to mature forms (Wolin and Matera 1999; Hopper and Phizicky 2003). However, how transcription and processing machines cooperate to ensure normal biosynthesis of tRNA in the cell remains essentially unknown.

RNA polymerase (Pol) III transcribes tRNA and other small, noncoding RNA genes in human cells. Transcription of tRNA genes by Pol III has been extensively studied in terms of promoter requirement and subunit recruitment (see Geiduschek and Kassavetis 2001; Schramm and Hernandez 2002; Roeder 2003; White 2005). tRNA genes contain type 2 promoters that are recognized by the transcription initiation factor TFIIC that recruits TFIIB, and then both factors assist the joining of Pol III to form a preinitiation transcription complex (Schramm and Hernandez 2002; Roeder 2003). 5S rRNA genes have type 1 promoter while U6 snRNA and 7SL RNA (SRP RNA) genes possess type 3 promoters, which are bound by distinct sets of factors that recruit Pol III (Schramm and Hernandez 2002; Roeder 2003).

Newly transcribed precursor transcripts of tRNA undergo processing and modifications through several steps, which include the removal of the 5′ leader sequence, elimination of the 3′ trailer sequence, splicing of introns in some tRNA isotypes, addition of CCA, and modification of nucleotides (Wolin and Matera 1999; Hopper and Phizicky 2003). Some of these steps of tRNA biogenesis are spatially and temporally ordered (Hopper 1998; Wolin and Matera 1999; Phizicky 2005) and regulated by the La protein (Intine et al. 2002), which has been shown to bind to chromatin of tRNA genes (Fairley et al. 2005).

Removal of the 5′ leader sequence of precursor tRNA is carried out by ribonuclease P (RNase P), a ubiquitous ribonucleoprotein endonuclease (Altman 2000). Bacterial RNase P has a catalytic RNA moiety that is associated with a single protein cofactor (Guerrier-Takada et al. 1983). Eukaryal RNase P ribonucleoproteins are large particles, when compared with their bacterial counterparts (Jarrous and Altman 2001; Xiao et al. 2002). The catalytically active form of human nuclear RNase P consists of an RNA component, termed H1 RNA, and at least 10 distinct protein subunits, designated Rpp14, Rpp20, Rpp21, Rpp25, Rpp29, Rpp30, Rpp38, Rpp40, hPop1, and hPop5 (see Jarrous and Altman 2001; van Eenennaam et al. 2001). Most of these proteins are shared with the mitochondrial and rRNA-processing ribonucleoprotein RNase MRP (Clayton 2001; van
Eenennaam et al. (2001). In fact, H1 RNA and its protein subunits are differentially concentrated in distinct intranuclear compartments—e.g., nucleolus, nucleoplasm, and Cajal bodies (Jarrous et al. 1999; van Eenennaam et al. 2001)—in which it is suggested that transcription and processing machines are assembled and modified (Gall 2003). Since these intranuclear compartments are known to be associated with specialized transcriptional activities and their structural integrity is dependent on continuous RNA synthesis (Lamond and Earnshaw 1998; Pombo et al. 1999), we have proposed that the assembly and function of human RNase P are linked to gene transcription (Jarrous 2002).

We show here that human RNase P is required for Pol III transcription of tRNA as well as other small, noncoding RNA genes in whole HeLa extracts and in cells. RNase P acts as a catalytic ribonucleoprotein in Pol III transcription and exerts its role through association with Pol III and chromatin of active tRNA and 5S rRNA genes. Our study reveals a role for a catalytic ribonucleoprotein—i.e., RNase P in Pol III transcription—and suggests that tRNA gene expression may be coordinated at transcription and processing levels.

Results

RNase P activity associates with core and specific subunits of Pol III

Coimmunoprecipitation studies revealed that RNase P activity, identified by processing of the 5' leader sequence of a 32P-labeled precursor tRNA\(^{\text{TYR}}\) [see Materials and Methods], was brought down from HeLa cells by the use of monoclonal antibodies directed against RPB6 (also known as HsRPABC2/RPB14.4) and RPB8 (HsRPABC3/RPB17) [Fig. 1A, lanes 3–7], two core protein components of Pol I, II and III [Woychik et al. 1990]. This coimmunoprecipitation of RNase P activity was sensitive to increasing concentrations of salt [Fig. 1B, cf. lanes 3,5 and 4,6]. An affinity-purified monoclonal antibody directed against RPC32 [also known as HsRPC7; Jones et al. 2000], a specific protein component of Pol III that is required for promoter-dependent initiation of transcription [Wang and Roeder 1997] also brought down RNase P activity from HeLa cells [Fig. 1A, lane 8]. Similar results were obtained using a monoclonal antibody directed against RPC39 (HsRPC6) [Fig. 1C, lanes 3–5], another specific subunit of Pol III [Wang and Roeder 1997]. An antibody directed against Bdp1, a subunit of TFIIB, also brought down RNase P activity [Fig. 1C, lanes 6,7]. The anti-RPC32, anti-RPC39, and anti-Bdp1 antibodies were used at optimal quantities since the use of excess amount of these antibodies in coimmunoprecipitation assays was not more effective in bringing down RNase P activity [Fig. 1D]. As positive control for immunoprecipitation [IP] of RNase P, we utilized polyclonal antibodies directed against Rpp21 [Fig. 1B [lanes 7,8], C [lane 8]]. Rpp21 is a specific subunit of active RNase P [Fig. 1F, lanes 5,6] and not shared with RNase MRP, as determined by Northern blot analysis of immunoprecipitated RNAs [Fig. 1E]. Thus, antibodies directed against endogenous Rpp21 and myc-tagged Rpp21 expressed in transfected HeLa cells mainly brought down the 340-nucleotide [nt] H1 RNA subunit of human RNase P [Fig. 1E, lanes 4,6,7], while antibodies against other Rpp subunits precipitated the 265-nt RNase MRP RNA as well [Fig. 1E, lanes 3,5].

The results described above show that RNase P activity can be coimmunoprecipitated with protein subunits of human Pol III.

RNase P is required for tRNA gene transcription in vitro

Transcription of an intron-containing human tRNA\(^{\text{TYR}}\) gene [Fig. 2A; van Tol et al. 1987] was carried out in whole HeLa extracts [Baer et al. 1990] subjected to immunodepletion analysis [see Materials and Methods]. In control extracts that were incubated with preimmune serum or Protein A/G agarose beads, transcription of the tRNA\(^{\text{TYR}}\) gene produced a 112-nt primary transcript that was promptly spliced to a stable 92-nt tRNA\(^{\text{TYR}}\) intermediate, which was subsequently processed at both termini to produce the mature 76-nt tRNA\(^{\text{TYR}}\) [Fig. 2B, lanes 5–7,11; see van Tol et al. 1987]. By contrast, a severe reduction of ∼90% in the synthesis of tRNA\(^{\text{TYR}}\) was measured in extracts immunodepleted with polyclonal anti-Rpp21 antibodies [Fig. 2B, lane 4], when compared with tRNA\(^{\text{TYR}}\) synthesis in extracts treated with preimmune serum [Fig. 2B, lane 7]. A comparable reduction in tRNA\(^{\text{TYR}}\) synthesis was observed in an extract immunodepleted with a monoclonal antibody directed against RPC32 [Fig. 2B, lane 10].

Transcription of the tRNA\(^{\text{TYR}}\) gene can be restored in the RNase P-depleted extract by adding back its corresponding immunoprecipitate [Fig. 2B, cf. lanes 8 and 4]. Western blot analyses detected RPB8 and RPC39 in the immunoprecipitates of anti-Rpp21 antibodies [Fig. 2D, cf. lanes 1–3 and 7], even though the majority of these two subunits remained in the immunodepleted extracts [IDs] [Fig. 2D, lanes 1–6]. This latter finding suggests that a small fraction of RNase P activity in the extract was associated with Pol III. We could not detect RPC32 in the immunoprecipitates of anti-Rpp21 antibodies [Fig. 2D, lanes 1–3, third panel], possibly because this subunit is less abundant than RPC39 [see Wang and Roeder 1997].

Inhibition of transcription in whole HeLa extracts immunodepleted of RNase P activity is not restricted to the tRNA\(^{\text{TYR}}\) gene. Thus, depletion of RNase P activity from extracts using anti-Rpp21 antibodies resulted in inhibition of the synthesis of human tRNA\(^{\text{MET}}\) [Fig. 2E, cf. lanes 3 and 5; tRNA\(^{\text{MET}}\)]. This deficiency could be alleviated by adding back the immunoprecipitated material to the depleted extract [Fig. 2E, cf. lanes 6 and 3; pRNA\(^{\text{MET}}\)]. Nonetheless, the newly transcribed primary transcripts of tRNA\(^{\text{MET}}\) were not processed to mature 75-nt tRNA\(^{\text{MET}}\) [Fig. 2E, lane 6]. Since this observation was reproduced with precursor transcripts of tRNA\(^{\text{TYR}}\)
the immunodepletion procedure of RNase P caused defect in the 5' tRNA processing capability of the reconstituted extract.

Depletion of whole HeLa extracts from RNase P activity by the use of polyclonal antibodies directed against Rpp40, another protein subunit of human RNase P [Jarrous and Altman 2001], caused a marked decline of ~90% in transcription of the tRNA^{Tyr} gene (Fig. 2F, lanes 3, 4) when compared with transcription in control extracts (Fig. 2F, lanes 7, 8). As expected, extracts treated with monoclonal antibodies directed against RPC32 and RPC39 showed severe reductions in transcription of the tRNA^{Tyr} gene (Fig. 2F, lanes 5, 6, respectively).

Our coimmunoprecipitation analyses reveal that the association of RNase P activity with Pol III components was sensitive to increasing concentrations of salt [Fig. 1B]. This observation was exploited to show that depletion of RNase P and not its associated Pol III components is responsible for inhibition of tRNA gene transcription in whole HeLa extracts. Thus, we performed immunodepletion of RNase P activity from whole HeLa extracts in the presence of 0.1, 0.2, and 0.3 M KCl [Fig. 3B, lanes 3–5], and the resultant immunodepleted extracts were dialyzed and tested for tRNA^{Met} gene transcription [Fig. 3A]. The dialysis step was included to eliminate the inhibitory effect of...
high concentrations of KCl on Pol III transcription [Fig. 3A, lanes 8,9]. We found that when compared with extracts treated with preimmune serum, those immunodepleted of RNase P activity by anti-Rpp29 antibodies [Fig. 3B, lanes 3–5] were all deficient in transcription of the tRNA^Met gene regardless of salt concentration [Fig. 3A, cf. lanes 1–3 and 4–6], though the control extract treated with 0.3 M KCl exhibited a decrease in Pol III transcription in spite of dialysis [Fig. 3A, cf. lanes 6 and 4,5].
RNase P is required for transcription of various small, noncoding RNA genes by Pol III

Whole HeLa extracts were subjected to immunodepletion analysis using antibodies directed against distinct protein subunits of human RNase P, and the immunodepleted extracts were then assayed for transcription of human 5S rRNA, 7SL RNA, and U6 snRNA genes. Sharp declines in transcription of 5S rRNA, 7SL RNA, and U6 snRNA genes were evident in extracts immunodepleted with antibodies directed against the subunits Rpp20, Rpp21, and Rpp29 when compared with transcription in extracts treated with antibodies against the tumor suppressor gene p53 or preimmune serum (Fig. 3D–F, cf. lanes 1–3 and 5,6). As expected, extracts immunodepleted with anti-RPC32 antibody were deficient in transcription of these three small, noncoding RNA genes (Fig. 3D–F, lane 4).

The results described so far demonstrate that a multiprotein complex of human RNase P is required for efficient transcription of various small, noncoding RNA genes by Pol III.

Reconstitution of Pol III transcription deficiency in extracts by exogenous RNase P

To test that a proficient Pol III exists in RNase P-depleted extracts, we added a partially purified HeLa RNase P, which was obtained from a DEAE anion-exchange chromatography column (see below; Jarrous and Altman 2001), and performed reconstitution of transcription of various small, noncoding RNA genes in RNase P-depleted extracts. Remarkably, the addition of a DEAE-purified RNase P (fraction F31; see below) restored the synthesis of 299-nt 7SL RNA transcript and 89-nt primary transcript of tRNA^{Met} in whole HeLa extracts immunodepleted of RNase P activity with antibodies directed against Rpp21 or Rpp29 (Fig. 4A, cf. lanes 3,5 and 2,4). Similarly, the addition of immunodepleted extracts from growing HeLa cells, subjected to immunodepletion using antibodies directed against Rpp20 (lane 1), Rpp21 (lane 2), Rpp29 (lane 3), RPC32 (lane 4), p53 (lane 5), or preimmune serum (lane 6), and then the immunodepleted extracts were split and analyzed for transcription of 5S rRNA or 7SL RNA and U6 snRNA (see Materials and Methods). The resultant 120-nt 5S rRNA, 299-nt 7SL RNA, and 106-nt U6 snRNA were all analyzed in the same 8% sequencing gel. E and F represent the upper and lower parts of the same gel with different exposures. Different exposures of the DNA size marker are attached to each panel. Asterisk points to labeled tRNA^{His}, and arrowhead indicates an aberrant U6 snRNA transcript.
tated RNase P to its corresponding immunodepleted extract resumed transcription of these two genes [Fig. 4B, cf. lanes 1, 2 and 3, 4]. However, the addition of a mini-RNase P—which has only three components, Rpp21 and Rpp29, and H1 RNA (Mann et al. 2003)—failed to restore transcription (Fig. 4A, lanes 2, 4), possibly because it lacks subunits found in the purified RNase P.

We noticed that the elution profile of active RNase P from the DEAE anion exchange chromatography column [Fig. 4D] described above partially overlapped with those
of Pol III components; e.g., RPB8, RPC32, and RPC39 (Fig. 4E). Hence, we performed reconstitution assays of Pol III transcription in RNase P-depleted whole HeLa extracts using fractions F31 and F36 with comparable RNase P activity [Fig. 4D]. Both fractions resumed 5S rRNA gene transcription in the immunodepleted extracts (Fig. 4F, cf. lanes 3, 6 and 7), even though fraction F36 was enriched with RPB8, RPC32, or RPC39 when compared with fraction F31 (Fig. 4E). To provide direct evidence that RNase P is responsible for restoration of 5S rRNA gene transcription, the H1 RNA moiety of RNase P in fractions F31 and F36 was targeted for cleavage by RNase H digestion using an antisense H1-1 deoxyoligonucleotide [see below]. The treated fractions failed to restore 5S rRNA gene transcription when compared with untreated ones (Fig. 4F, cf. lanes 1, 4 and 3, 6). Reconstitution of transcription was observed when fractions F31 and F36 were incubated with a scrambled H1-1 deoxyoligonucleotide, termed H1-1sc and RNase H (Fig. 4F, cf. lanes 2, 5, and 7).

As noted above, whether reconstitution of Pol III transcription was done with endogenous RNase P or DEAE-purified RNase P, newly transcribed 89-nt primary transcripts of tRNA<sup>Met</sup> were not processed to 75-nt mature tRNA<sup>Met</sup> [Fig. 4A (lanes 3, 5), B (lane 2)]. To verify that the DEAE-purified RNase P and the mini-RNase P used in the aforementioned reconstitution assays were functional in tRNA processing, we added them to IgG-treated (Fig. 4A, lanes 6, 7) and untreated (Fig. 4B, lanes 5–8) whole HeLa extracts. In both cases, the DEAE-purified RNase P (fraction F31) enhanced transcription of the 7SL RNA and tRNA<sup>Met</sup> genes and accelerated the maturation of tRNA<sup>Met</sup> [Fig. 4A, B, cf. lanes 7 and 8]. The mini-RNase P had no effect on transcription of these genes, but it did elicit formation of 75-nt tRNA<sup>Met</sup> [Fig. 4A, cf. lanes 6 and 8], a finding that is in agreement with reconstitution experiments showing that the mini-RNase P has endonucleolytic activity in vitro [Mann et al. 2003].

**Targeted cleavage of H1 RNA alters enzyme specificity of RNase P and diminishes Pol III transcription**

Several antisense deoxyoligonucleotides designed to anneal to the catalytic and specificity domains of H1 RNA [Fig. 5A], including the H1-1 deoxyoligonucleotide described above, were tested for targeted inactivation of RNase P in whole HeLa extracts by the use of RNase H [see Materials and Methods]. Two of these deoxyoligonucleotides, H1-1 and H1-8 [Lee et al. 1996], were found to mediate cleavage of synthetic H1 RNA transcripts by RNase H [Fig. 5B, lanes 6, 7]. In whole HeLa extracts preincubated with RNase H and H1-1 deoxyoligonucleotide, transcription of the 5S rRNA gene was reduced by up to ~70% [Fig. 5C, lanes 4–6] when compared with transcription in the control extract incubated with the H1-1 deoxyoligonucleotide alone [Fig. 5C, lane 2]. A reduction of ~40% in gene transcription was measured in extracts incubated with H1-8 deoxyoligonucleotide and RNase H [Fig. 5C, cf. lanes 7–9 and 3]. Treatment of extracts with RNase H and H1-1 but not scrambled H1-1sc deoxyoligonucleotide caused severe reductions in transcription of several small, noncoding RNA genes, including 7SL RNA [Fig. 5E, cf. lanes 2 and 3], 5S rRNA [Fig. 5E, cf. lanes 4 and 5], tRNA<sup>Tyr</sup> [Fig. 5E, cf. lanes 6 and 7], and tRNA<sup>Met</sup> [Fig. 5E, cf. lanes 8 and 9] genes. In the case of the 7SL RNA gene, a notable reduction of ~95% in its transcription was measured in extracts treated with H1-1 versus H1-1sc [Fig. 5E, cf. lanes 2 and 3]. Since the H1-1, but not H1-1sc, deoxyoligonucleotide is designed to anneal to H1 RNA, the former deoxyoligonucleotide can affect H1 RNA conformation and function. This can explain the large inhibitory effect of the H1-1 deoxyoligonucleotide on 5S rRNA gene transcription when it was compared with H1-1sc [Fig. 5E, cf. lanes 4 and 5] and not H1-1 [Fig. 5C, cf. lanes 6 and 2].

A defect in RNase P activity, as manifested by incorrect cleavage of the 5’ leader sequence of the *Schizosaccharomyces pombe* precursor tRNA<sup>Met</sup> [pSupS1] was seen in extracts incubated with RNase H and H1-1 or H1-8 deoxyoligonucleotide [Fig. 5D, lanes 5–10; arrow]. By contrast, extracts treated with H1-1 or H1-8 deoxyoligonucleotide without RNase H exhibited accurate cleavage of this substrate by RNase P [Fig. 5D, lanes 3, 4]. Similarly, miscleavage of the *Escherichia coli* tRNA<sup>Tyr</sup> precursor tRNA<sup>Tyr</sup> by RNase P was also evident in whole HeLa extracts treated with RNase H and H1-1 [Supplementary Fig. 1, lane 4] or H1-8 [Supplementary Fig. 1, lane 3] deoxyoligonucleotide.

Since the H1-1 and H1-8 deoxyoligonucleotides target the specificity domain of H1 RNA [Fig. 5A], which is implicated in substrate recognition by RNase P [Mann et al. 2003], the findings described above support the notion that properly active RNase P is required for transcription of small, noncoding RNA genes carrying the three basic types of Pol III promoters.

**RNase P is required for Pol III transcription in the cell**

HeLa cells at ~40% confluence were transiently transfected with siRNA38 [see Materials and Methods], a small interfering RNA (siRNA) shown to target the subunit Rpp38 of human RNase P [Cohen et al. 2003], and whole-cell extracts were prepared at various time points after transfection. An efficient knockdown of Rpp38 was measured in siRNA38-transfected cells when compared with control cells [Fig. 6A, cf. lanes 1–3 and 4–6], while expression of the subunit Rpp40 as well as β-actin was not affected [Fig. 6B, C]. This targeted knockdown of Rpp38 was accompanied by a marked reduction in the activity of RNase P in tRNA processing [Fig. 6D]. Strikingly, while the 5S rRNA gene was transcribed in extracts obtained from untransfected cells [Fig. 6E, lanes 5, 6], no transcription was seen in extracts prepared from the siRNA38-treated cells [Fig. 6E, lanes 2, 3]. The lack of transcription in the control extract prepared from cells harvested at 66 h after transfection [Fig. 6E, lane 7] was
due to cessation of cell proliferation in the 3-d-old culture (see below).

To corroborate the role of RNase P in Pol III transcription, we targeted another subunit, Rpp29, for destruction by siRNA29 in transiently transfected HeLa cells. This siRNA, which was first described by others (Zhang and Altman 2004), knocked down Rpp29 by $\sim 95\%$ when compared with its expression in cells transfected with a luciferase siRNA (Fig. 6F, cf. lanes 4–6 and 1–3). Whole extracts obtained from these siRNA29-transfected cells at 22, 44, and 66 h after transfection were defective in 5S rRNA gene transcription (Fig. 6J, lanes 1–3). By contrast, whole extracts prepared from cells transfected with a luciferase siRNA showed active transcription of the 5S rRNA gene (Fig. 6J, lanes 4–6).

To show that RNase P is required for Pol III promoter activity in living cells, HeLa cells were transfected with siRNA38 in the presence or absence of pU6-M1 RNA, in which a bacterial reporter gene encoding the M1 RNA subunit of $E. coli$ RNase P (Guerrier-Takada et al. 1983) was abutted to a mouse U6 snRNA promoter (see Materials and Methods). Total RNA was extracted from cells, which were analyzed for RNase P activity (Fig. 7D, lanes 3–6), and expression of M1 RNA was determined by ribonuclease protection analysis (Fig. 7A) and Northern blot analysis (Fig. 7B) using an antisense, $^{32}$P-labeled M1 RNA as probe. While mock-transfected cells expressed high levels of full-length M1 RNA (377 nt in length) (Fig. 7A,B, lanes 4,5), expression of this transcript was reduced by $\sim 90\%$ in siRNA38-treated cells (Fig. 7A,B [lanes 2 and 3], C). By contrast, siRNA38 had no effect on expression of a green fluorescent protein gene fused...
to a Pol II promoter [e.g., CMV promoter] in cotransfected HeLa cells [data not shown]. The different time courses for the effect of siRNA38 on the expression of M1 RNA in cells (Fig. 7A, lanes 2,3) versus 5S rRNA transcription in extracts (Fig. 6E, lanes 2,3) could be due to the time required for siRNA38 to work effectively in cotransfection procedures. Thus, the low expression of M1 RNA detected at 25 h (Fig. 7A, lane 2) could be due to the persistence of stable M1 RNA transcripts synthesized at earlier time after cotransfection of cells.

The findings described above reveal that inactivation of RNase P by RNA interference (RNAi) abolishes Pol III transcription and Pol III-directed promoter activity in the cell.

A multiprotein RNase P complex associates with chromatin of tRNA and 5S rRNA genes

Since RNase P is needed for Pol III transcription, we tested if it can associate with actively transcribed non-coding RNA genes by the use of chromatin immunoprecipitation (ChIP) analysis [see Materials and methods]. Thus, ChIP assays were performed with HeLa cells using various antibodies directed against RNase P and Pol III subunits, and the immunoprecipitated chromatin materials were examined for enrichment of specific genes by PCR [see Materials and Methods]. A PCR signal for a human tRNA\(^{Tyr}\) gene was detected in chromatin samples precipitated by antibodies directed against Rpp14, Rpp21, Rpp30, and Rpp40 [Fig. 8A, lanes 1–4, respectively]. This tRNA\(^{Tyr}\) gene was also enriched in chromatin preparations brought down by anti-RPB8 and anti-RPC32 antibodies [Fig. 8A, lanes 5 and 6] but not by preimmune rabbit serum or beads alone [Fig. 8A, lanes 7,8]. PCR signals for tRNA\(^{Leu}\) and 5S rRNA genes were also detected in these chromatin preparations, except for the one obtained with the anti-Rpp30 antibody [Fig. 8A, lane 3; second and last panels], which could have failed to recognize its corresponding subunit in the chromatin context of these genes. The 7SL RNA gene was identified.
in chromatin samples precipitated by the anti-RPB8 antibody [Fig. 8A, lane 5].

The association of Rpp21 and Rpp29 with chromatin of tRNA\textsubscript{ Tyr}, tRNA\textsubscript{ Leu}, and 5S rRNA genes increased by fourfold to 10-fold when HeLa cells grew from 30% to 60% confluence (Fig. 8B, cf. lanes 1, 2, and 6, 7) but then decreased when cells reached 95% confluence (Fig. 8B, lanes 10, 11), in which proliferation of the cells declined as determined by flow cytometry (data not shown). Rpp21 and Rpp29 did not bind to chromatin of the 7SL RNA and β-globin genes (Fig. 8B, lanes 1, 2, 6, 7). The binding pattern of Rpp21 and Rpp29 to chromatin of the tRNA\textsubscript{Tyr}, tRNA\textsubscript{Leu}, and 5S rRNA genes resembled that of RPB8 (Fig. 8C–E), a core component of active Pol III. The increase in chromatin binding by these protein subunits of RNase P and Pol III in cells at 60% confluence (Fig. 8C–E) coincided with vigorous Pol III transcription seen in extracts obtained from cells with a comparable confluence (data not shown). However, Rpp29 disengaged from tRNA and 5S rRNA genes independently of Rpp21 and RPB8 in cells at 95% confluence (Fig. 8B, cf. lanes 11 and 10, 12). In addition, knockdown of Rpp29 in cells by the use of siRNA29 selectively eliminated the chromatin binding signal of Rpp29 but not that of RPB8 and Rpp21 to tRNA and 5S rRNA genes (Fig. 8F, cf. lanes 2 and 1, 3). Rpp29 occupancy of these genes remained unchanged in cells transfected with a control, luciferase siRNA (Fig. 8F, lane 7). Thus, recruitment of Pol III (represented by RPB8) to tRNA and 5S rRNA genes, which occurs under conditions of inhibition of Pol III transcription in cells with knockdown of siRNA29 [Fig. 8G], does not require a functional RNase P.

RNase P dissociates from chromatin of inactive 5S rRNA genes in mitotic cells

To provide evidence that the association of RNase P subunits with chromatin of small, noncoding RNA genes is linked to active transcription, HeLa cells were synchronized to G2 and M phase by treatment with nocodazole, and then G2-enriched and mitotic cell populations [see Supplemental Material; White et al. 1995] were subjected to FACS analysis for determination of their DNA content (Supplementary Fig. 2A–C). Whole extracts were prepared from these two synchronized cell populations and tested for Pol III transcription (Supplementary Fig. 2D). Transcription of the 5S rRNA gene was inefficient in the mitotic extract when compared with transcription in the G2 extract (Supplementary Fig. 2D, cf. lanes 2 and 1), a finding that is consistent with a previous study [White et al. 1995]. A decrease of ∼50% in RNase P activity in processing of a precursor tRNA\textsubscript{Tyr} was also evident in the mitotic extract (Supplementary Fig. 2E, cf. lanes 3 and 4). ChIP analysis revealed that Rpp20 and Rpp29 were not bound to chromatin of the 5S rRNA genes in mitotic cells, when compared with cells at G2 phase (Supplementary Fig. 2E, cf. lanes 3 and 4). Rpp21 and RPB8 occupancy of 5S rRNA genes was also considerably reduced (Supplementary Fig. 2G, cf. lanes 2, 4, and 7, 9). This substantial decrease in chromatin binding by RPB8, which represents active Pol III, supports the weak in
vitro transcription capability of the mitotic extract (Supplementary Fig. 2D).

Taken together, the results described above reveal that a multiprotein complex of human RNase P is associated with chromatin of transcriptionally active small, noncoding RNA genes. In addition, this associa-
Mechanism of action of RNase P in Pol III transcription

Protein subunits of human and yeast nuclear RNase P do not interact with components of the transcription machineries, as assessed by yeast two-hybrid genetic screens [Jiang and Altman 2001; see Xiao et al. 2002], though future studies that involve pull-down assays [Hu et al. 2004] could help in revealing weak protein–protein interactions between RNase P and Pol III subunits. Nonetheless, the association of RNase P with Pol III in the cell could be facilitated by a third partner, e.g., tRNA gene. Our ChIP analyses uncover a multiprotein complex of RNase P that is associated with chromatin of transcriptionally active tRNA and 5S rRNA genes. RNase P subunits are maximally bound to chromatin of tRNA and 5S rRNA genes in dividing cells [Fig. 8] while dissociate from chromatin in mitotic cells [Supplementary Fig. 2]. Hence, the association of RNase P and Pol III with chromatin of these target genes correlates with cell proliferation and cell cycle. Our results demonstrate that these two large molecular machines are associated in the context of chromatin, thus providing a mechanistic explanation of how RNase P exerts its role in Pol III transcription. However, knockdown of Rpp29 by RNAi does not eliminate binding of Pol III [represented by its core protein RPB8] to tRNA and 5S rRNA genes [Fig. 8F]. In addition, Rpp29 dissociates from chromatin of these genes independently of RPB8 [Fig. 8F]. Thus, Pol III occupancy of tRNA and 5S rRNA genes is not dependent on the presence of a functional RNase P in the cell. Whether Pol III or its transcription factors rather recruit RNase P on these target genes awaits thorough analysis.

In contrast to tRNA and 5S rRNA genes, the 7SL RNA and possibly U6 snRNA genes seem not to be bound by RNase P subunits as determined by ChIP analysis [Fig. 8]. This latter finding suggests that RNase P has two modes of action in Pol III transcription. First, Pol III requires RNase P for general transcription, but the latter particle does not intimately bind to all genes. Second, RNase P binds to chromatin of genes that their nascent transcripts serve as its substrates; e.g., tRNA and probably 5S rRNA precursors.

RNase P, Pol III, and transcription factories

Coordination of the activities of several macromolecular machines involved in transcription, processing, and export of mRNA has been described [see Maniatis and Reed 2002; Proudfoot et al. 2002]. These activities are coordinated in complex and extensive coupling networks that support the existence of “Pol II transcription factories” in the cell [see Cook 2002; Maniatis and Reed 2002]. These networks become knotty as some of their components are also involved in tRNA processing [Paushkin et al. 2004]. In S. cerevisiae, it has been shown that dispersed tRNA genes can cluster in a transcription-dependent manner in the nucleolus [Thompson et al. 2003], which could serve as a Pol III transcription factory. Similarly, biochemical and RNA cytochemistry experiments with human cells unveil the possible existence of Pol III
transcription factories in the nucleoplasm (Pombo et al. 1999). Through mechanisms of chromatin mobility (Kosak and Groudine 2004; Osborne et al. 2004), tRNA genes from discrete genomic loci could be put close in nucleoplasmic or nucleolar regions enriched with macromolecular assemblies of transcription and processing machines, including Pol III and RNase P, for their coordinated expression at multiple levels.

Materials and methods

Whole HeLa extracts, immunodepletion, and transcription

HeLa cells were grown in 12–24 flasks of 175 cm² and harvested at confluences specified in each experiment. Fifty percent confluency was obtained at ~1 x 10⁶ cells per flask. Adhered cells were detached by trypsin treatment followed by centrifugation. Whole HeLa extracts were then prepared as described by Baer et al. [1990]. Protein concentrations in extracts were 10–15 mg/mL. For each immunodepletion assay, 25 µL of extract diluted by 1:1 to 1:2 in dialysis buffer was incubated with an antibody coupled to Protein A/G agarose beads (100–300 µL serum with 40–50 µL slurry beads). After 3 h of incubation at 4°C, beads were centrifuged and supernatants were collected and assayed for RNase P activity and for Pol III transcription. The corresponding immunoprecipitates were washed three times with 1 x NET2 buffer containing 150 mM NaCl and three times with 1 x PA and assayed for RNase P activity.


ChIP and PCR analysis

ChIP analysis was done essentially as described by Gomez-Roman et al. (2003). Adherent HeLa cells were grown to various confluences, collected, washed with 1 x PBS, and cross-linked with 0.5% NP-40/1 x PBS containing 1% formaldehyde for 10 min at 37°C. Cells were rinsed with ice-cold 0.5% NP-40/1 x PBS and incubated for 30 min in high-salt buffer (1 M NaCl, 0.5% NP-40, 1 x PBS). Cells were collected, washed with 0.5% NP-40/1 x PBS, and then resuspended in low-salt buffer (0.1 M NaCl, 0.1% NP-40, 10 mM Tris-Cl at pH 8.0, 1 mM EDTA). After 30 min, cells were centrifuged at 480 x g for 10 min and subjected to 10 strokes through a 23-gauge needle. Nuclei obtained after centrifugation were resuspended in low-salt buffer containing 2% sarkosyl and transferred to a sucrose cushion (0.1 M sucrose, 0.1 M NaCl, 10 mM Tris-Cl at pH 8.0, 1 mM EDTA, 0.5% NP-40), and then spun for 10 min at 4000 x g. The pellet was resuspended in TE (10 mM Tris-Cl at pH 8.0, 1 mM EDTA) and spun again, and genomic DNA was sheared by sonication [14 x 10 sec, duty cycle 30%] to produce stretches of chromatin of 400–1000 base pairs [bp] in length. The sonicated material was diluted in 1/10 volume of x11 NET buffer (1.65 M NaCl, 550 mM Tris-Cl at pH 7.4, 5.5 mM EDTA, 5.5% NP-40).

The sonicated material (~1 mL volume) was precleared for 30 min with 20 µL of Protein A/G Plus agarose beads [Santa Cruz Biotechnology] first blocked with 2 µg of salmon sperm [Invitrogen]. After centrifugation, 0.1-mL samples were subjected to IP overnight at 4°C using a mutating device with the appropriate antibody coupled to beads (coupling was for 6 h at 4°C) and in the presence of 4 µg of salmon sperm. Precipitated complexes were washed three times with 1 mL of RIPa buffer [80 mM Tris-Cl at pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40], three times with 1 mL of LiCl buffer [10 mM Tris-Cl at pH 8.0, 250 mM LiCl, 1 mM EDTA 0.5% deoxycholate, 0.5% NP-40], and then with 3 mL of TE. Immunoprecipitated material was eluted twice with 200 µL of 1% SDS/TE and incubated overnight at 42°C in 0.4 mL volume of elution buffer containing 120 µg/mL Proteinase K DNA. Extracted was extracted twice with phenol:chloroform:isoamyl alcohol [25:24:1 v:v], ethanol-precipitated, washed with 70% ethanol, dried, and resuspended in 25 µL distilled water for use in PCR analysis.

Each PCR reaction contained 3 µL of primers [final concentration of 1 µM], 5 µL of reaction mixture, and 4 µL of DNA for each IP sample. Input DNA was diluted in distilled water before PCR. PCR amplification programs and primer sequences were described in Winter et al. [2000] and Gomez-Roman et al. [2003] (see Supplemental Material).

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References


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References
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