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Pol II and its associated epigenetic marks are present at pol III-transcribed non-coding RNA genes

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Abstract

Epigenetic control is an important aspect of gene regulation. Despite detailed understanding of protein-coding gene expression, the transcription of non-coding RNA genes by RNA polymerase (pol) III is less well characterized. Here we profile the epigenetic features of pol III target genes throughout the human genome. This reveals that the chromatin landscape of pol III-transcribed genes resembles that of pol II templates in many ways, although there are also clear differences. Our analysis also discovered an entirely unexpected phenomenon, namely that pol II is present at the majority of genomic loci that are bound by pol III.

Whereas prokaryotes and archaea use a single RNA polymerase to transcribe their genomes, this function is divided in eukaryotes between several specialized enzymes1. The largest of these is pol III, which has 17 subunits, all of which are required for cell viability. Pol III is responsible for ~10% of all nuclear transcription and makes short non-coding RNAs, including tRNA and 5S rRNA1. Other essential pol III transcripts include 7SL RNA, which participates in protein trafficking, and U6, RNase P and MRP RNAs, that are required for processing mRNA, tRNA and rRNA, respectively1. MRP RNA also interacts with the catalytic subunit of telomerase reverse transcriptase2. In addition, pol III synthesizes 7SK RNA, that controls the pol II transcription elongation factor PTEF-b3,4, and HVG RNA, that forms part of the vault particles implicated in multidrug resistance5. Some studies also suggest that pol III may be involved in transcription of miRNA genes6,7.

Chromatin has been shown to exert a powerful influence on transcription by pols I and II8,9, while most attention has focused on the pol II system, particularly in documenting the global chromatin modification patterns associated with protein-coding genes10–12. This study aims to broaden our understanding to include the chromatin status of pol III templates in human cells. Our data reveal on a genome-wide scale that certain features of chromatin are

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shared between genes transcribed by pols II and III, while important distinctions are also observed between these two classes.

RESULTS

Epigenetic marks at human pol III-transcribed genes

We analyzed histone modifications in human $CD4^+$ T cells in the vicinity of pol III-transcribed genes. Epigenetic marks usually associated with active pol II transcription were detected at many, but not all pol III genes. For example, comparison of two tRNALeu-TAA loci on chromosome 6 revealed strong H3K4me1/2/3 at one, but not the other, whereas the converse pattern was seen for H3K27me3 (Fig. 1). To test if chromatin environment correlates with expression status of pol III genes, we characterized their expression in $CD4^+$ T cells using two approaches: RNA-Seq analysis of total RNAs and ChIP-Seq analysis of pol III binding. A difficulty here is that RNA-Seq reads derived from repetitive templates (like many pol III genes) are frequently unmappable in a unique manner. However, these genes are often flanked by unique sequences, which allows mapping of pol III occupancy as an indicator of gene activity. Although polymerase binding to a gene does not necessarily mean it is transcribed10,13,14, confidence can be gained from a strong correlation between the number of RNA-Seq tags mapped to tRNA genes located in non-repetitive loci and the number of pol III ChIP-Seq tags mapped in the vicinity (Spearman correlation R^2=0.796, p<2.2e-16).

RNA-Seq of total RNAs detected transcripts from the tRNALeu-TAA gene marked by H3K4me1/2/3, but not from the gene marked by H3K27me3 (Fig. 1a,b). Consistent with this, pol III was crosslinked strongly to the former but not to the latter. This suggests that H3K4 methylation correlates with tRNA expression, whereas H3K27 trimethylation correlates with repression, as is well-documented for pol II transcription10,15,16. To confirm this on a global level, we assessed 302 tRNA genes located in non-repetitive areas of the genome and found that pol III signals indeed correlate with H3K4me1/2/3/ac (Fig. 2a, left panel; Supplementary Fig. 1). For comparison, the chromatin features for pol II genes occupied or not by polymerase were also plotted (Fig. 2, right panels). Other histone methylation and acetylation signatures also correlate with activity of tRNA genes, including H3K9ac, H3K23ac, H3K27ac, H3K36ac and others. In addition, active tRNA genes are enriched in H2A.Z and various histone acetylations. In contrast, levels of H3K27me2/3 and H3K9me3 are higher at the inactive tRNA genes (Fig. 2d, left panel, Supplementary Fig. 1).

Although these features are similar to those of pol II templates, some distinctions are also evident when the tag density profiles are compared (Fig. 2; Supplementary Fig. 1). A striking difference is that pol III templates lack enrichment for H3K79me2 and H3K36me3 (Fig. 2e–f), which are deposited within pol II-transcribed regions. In yeast, targeting of H3K36me3 is achieved by binding of Set2 methyltransferases to the phosphorylated CTD tail of elongating pol II17, a structure that is absent from pol III. Furthermore, several modifications, including H2AK9ac, H3K18ac, H3K27me1, H4K16ac and H4K20me1, are generally stronger at active than inactive tRNA genes, but do not show the clear peaks found at transcription start sites (TSS) of active pol II genes (Supplementary Fig. 1), suggesting

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that while expressed genes are located in a permissive chromatin environment, these modifications are not targeted to pol III genes specifically.

H3K4me1/2/3/ac, H3K9ac, H3K18ac and H2A.Z are found in nucleosomes located on both sides of a pol II TSS, especially the +1 nucleosome, whereas such modifications are strongest at the −1 nucleosome of tRNA genes and weaker after the TSS (Fig. 2a–c). It is likely that the weaker histone modification signals at the +1 nucleosome of tRNA genes is caused by nucleosome loss. Indeed, histone H3 ChIP-Seq experiments indicated enrichment of nucleosomes outside tRNA genes and a dramatic loss of histones within the tRNA genes bound by pol III, as compared to those not bound by pol III (Fig. 2g). However, we also observed some histone depletion at inactive tRNA genes, which may be explained by reduced mappability of ChIP-Seq reads at these sites. Alternatively, the continued presence of TFIIIC when transcription is low18,19 may exclude nucleosomes from tRNA-coding regions.

The chromatin features described above for tRNA genes were also observed at a variety of other pol III templates, including genes encoding U6 snRNA, 7SK, 7SL, hY1-5, HVG1-3, RNase P and MRP RNAs (Fig. 1c–f and data not shown). Among the 49 copies of U6 snRNA genes annotated in the human genome, we observed Pol III enrichment only at five loci (located at: chr14: 31740801, 31742026; chr15: 65919237; chr19: 844283, 972320). Also low levels of pol III were detected at chr1:27524096. ChIP-Seq was uninformative with regards to 5S rRNA genes because of their highly repetitive nature. Nevertheless, we conclude that a similar chromatin landscape is shared by a large proportion of disparate pol III templates.

Cell type-specificity of pol III gene expression

ChIP-Seq analysis of pol III in HeLa cells revealed similar patterns to $CD4^+$ T cells. Although the majority of tRNA genes are utilized in both cell types, a surprisingly large number (26%) of them were associated with pol III in only one of the cell types (Fig. 3a). This is consistent with a report that many tRNAs display tissue specificity20. An example is shown in Fig. 3b, and it is noteworthy that H3K4me3 levels at this site are much higher in $CD4^+$ T cells, where the gene appears active, than in HeLa cells, where pol III binding is not detected. In contrast to the tRNA genes, we did not find differences between $CD4^+$ T and HeLa cells in chromatin modifications or pol III occupancy at U6 genes.

Novel pol III targets

It has been suggested that pol III is responsible for expression of C19MC, the largest miRNA gene cluster known in humans6. This, however, has been challenged21. We did not detect expression of these miRNAs or pol III association in either $CD4^+$ T or HeLa cells, which are different cell types from those used in the previous work. A possible exception is MIR498, where a pol III peak was detected in $CD4^+$ T but not HeLa cells (not shown). However, this peak may be an artifact, as it did not coincide with pol III transcription factors (TFIIIB and TFIIIC, data not shown) or the chromatin modifications detected at confirmed sites of pol III transcription.

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We also examined the miRNA loci that were reported to bind pol III in melanoma and breast cancer cell lines7. However, only MIR565 showed pol III binding in $CD4^+$ T or HeLa cells (Supplementary table 2), even though several of these miRNAs were expressed in $CD4^+$ T cells22 (Supplementary Table 2). The exception, MIR565, is actually a tRNA fragment, which explains the localization of pol III there23. Our data therefore provide no evidence that miRNA loci are transcribed by pol III in HeLa and $CD4^+$ T cells.

To identify novel loci that might be transcribed by pol III, we identified pol III binding sites in HeLa cells using only U0 tags and SISSRs software18 and excluded sites that are located in the vicinity of known pol III genes and Repeatmasker annotated repeats. These are detailed in Supplementary Table 3. Interestingly, pol III was detected at 30 pol II gene promoters including MGC11102 (Fig. 4a). Novel pol III target sites were also detected either upstream of some pol II target gene promoters or in intergenic regions free of any known gene units (Fig. 4b,c). To assess further whether these are likely to be *bona fide* pol III transcription units, we carried out genome-wide ChIP-Seq analyses for the pol III-specific transcription factors TFIIIB and TFIIIC. TFIIIC is a DNA-binding factor that recognizes directly the promoters of most pol III-transcribed genes; it then recruits TFIIIB, which in turn recruits pol III and helps it initiate transcription. The co-existence of TFIIIB and TFIIIC with pol III in these regions (Fig. 4a,b,c) suggests that these sites have functional pol III promoters. Consistent with this hypothesis, transcripts were detected by the total RNA-Seq, but not by polyA RNA-Seq analyses (Fig. 4b,c). Given that polyA tail is a common feature of pol II transcripts, these data suggest that pol III might indeed transcribe these loci. Among the 177 novel sites bound by pol III, 124 loci were also bound by both TFIIIB and TFIIIC, while 23 and 12, respectively, were bound by individual transcription factors (Supplementary Table 3 and Fig. 4d). Total RNA-Seq signal was detected at 42% of these loci. Analysis of the TFIIIB and TFIIIC ChIP-Seq data using similarly stringent parameters revealed 111 and 185 novel binding sites located away from known pol III genes and repeats, respectively. Interestingly, the majority of these sites were also bound by pol III and the other TFIII factor (Fig. 4e,f, Supplementary Table 3). These data suggest that in addition to the known non-coding RNA genes, there is a large number of genomic regions that may be transcribed by pol III in the human genome.

Pol II associates with pol III-transcribed genes in human cells

Listerman et al.25 reported strong enrichment of pol II in the region 300–700 bp upstream of the start sites of active U6 snRNA genes. Our pol II ChIP-Seq data confirmed this observation and revealed that the phenomenon is widespread. In addition to U6 snRNA genes, pol II was found to be closely associated with a range of pol III-transcribed genes, including 7SK, MRP, HVG and a large proportion of tRNA genes in human $CD4^+$ T cells (Figs. 1 and 5). Fortuitous cross-reaction was excluded by the use of four alternative pol II antibodies (Fig. 5a). Pol II was also present at tRNA promoters in other human and mouse cell lines, including ES, hematopoietic stem cells and B cells (data not shown), as well as in *Drosophila* S2 cells (Fig. 5b), suggesting that the recruitment of pol II to pol III-transcribed genes is evolutionarily conserved. Similar results were obtained in HeLa cells by quantitative PCR-based ChIP assays (Fig. 5c and Supplementary Fig. 2). This approach also allowed detection of pol II at 5S rRNA genes (Fig. 5c). Additional components of the pol II

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transcription apparatus were detected at pol III templates, including TFIIB, TFIIE and TFIIH and the cyclin T1 subunit of PTEF-b (Fig. 5c; Supplementary Fig. 2). Crosslinking to these sites shows specificity, as it was not detected at the *ApoE* gene, which is not transcribed in these cells, or a "desert" region that lacks annotated gene sequences. In contrast, histone H3 could be crosslinked to each of the loci (Fig. 5c).

Additional evidence of specificity was provided by the pol I factor TAF_I 48, which was not detected at any site analyzed (Fig. 5c). Whereas the pol III signal peaked at the tRNA-coding region, as expected, peaks of pol II were typically found ~200 bp upstream of these genes (Supplementary Fig. 2). This was especially clear in ChIP-Seq tag density profiles (Fig. 5d). However, there was considerable overlap of pol II and pol III enrichment at these loci.

DISCUSSION

Histone modifications at pol III-transcribed genes

This study provides a detailed global analysis of the modification status of histones associated with pol III-transcribed genes. It shows a clear correlation between histone acetylation and apparent gene activity. A partial explanation is offered by evidence that tRNA and 5S or U6 genes are bound and induced by the GCN5 and p300 acetyltransferases19,26. These data are consistent with previous evidence that expression of tRNA and 5S rRNA genes can be stimulated by acetylation of core histones, both in vitro and in vivo19,26–31. In contrast, histone methylation had not previously been implicated as a major contributor to pol III regulation. Our analysis reveals that genes which are bound and expressed by pol III are marked by H3K4me1/2/3, whereas H3K9me3 and H3K27me2/3 correlate with apparent inactivity. Cause and effect are not distinguishable from these observations, so it will be important to test if the methylases responsible for these modifications are able to influence pol III transcription.

What brings pol II to pol III-transcribed genes?

We cannot yet explain the unexpected presence of pol II and its associated basal factors at pol III-transcribed genes. We found strong correlation between the detection of pols II and III at tRNA genes (Spearman R^2= 0.768, p<2.2e-16), suggesting that both polymerases are recruited to these sites by a common mechanism. Shared components of the transcriptional machinery might be involved. For example, TBP can nucleate assembly of a pol II preinitiation complex and is present at all pol III-transcribed genes as part of TFIIIB32. Most pol III templates also utilize c-Myc33, which might recruit pol II transcription factors, just as it brings GCN5 to tRNA and 5S rRNA genes19. It is likely that much more overlap remains to be discovered between transcription factors that have been regarded as being specific for pol II or pol III. For example, the pol II elongation factor TFIIS was recently found to stimulate pol III occupancy and transcription of tRNA genes in yeast34. A complex balance of interactions and influences may determine the occupancy of each individual site, depending on conditions.

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Significance of pol II at pol III-transcribed genes

It is unclear to what extent pol II transcribes DNA at the pol III loci. Pol II products derived from these sites have not been identified, but one cannot exclude transcripts that are too rare or short-lived to be detected. An argument against this is the apparent absence of H3K36me3 and H3K79me2, which are generally found in pol II-transcribed regions. However, we do detect pol II with its C-terminal domain phosphorylated at serine 2 and serine 5, modifications that occur after RNA synthesis commences. We can also cross-link cyclin T1, a component of the pol II elongation factor PTEF-b. Some level of pol II transcription initiation may therefore occur at these apparently ectopic sites, even if it is unproductive.

Listerman et al25 observed strong enrichment of pol II in the region upstream of the start sites of active U6 snRNA genes. They convincingly showed that low doses of α-amanitin reduced crosslinking of pol II and acetylation of histone H4 at these sites, as well as decreasing pol III-mediated synthesis of U6 snRNA25. On this basis, they speculated that pol II recruitment upstream of the U6 promoter might facilitate pol III transcription by modifying the local chromatin structure25. Our evidence for pol II slightly upstream of multiple pol III-transcribed genes, along with peaks of positive histone modifications, supports the idea that pol II might attract histone modifying enzymes to these loci. However, in contrast to U6 snRNA, Listerman et al. saw little or no effect on expression of other pol III transcripts when pol II was selectively inhibited with α-amanitin. Their observations suggest that pol III activity at a subset of loci may be affected by pol II, although indirect effects cannot be excluded. Our own experiments with short-term α-amanitin treatment (using higher concentration than that used by Listerman et al.) are also consistent with pol II activity being able to influence expression of some pol III products (Fig. 6). However, these data must be treated with great caution, as direct and indirect effects are very difficult to distinguish in vivo.

Pol III-transcribed genes have been shown to act as boundary elements in both yeast and mammals 38,39. In yeast, tRNA genes can serve as barriers to the propagation of heterochromatin40–43. This effect is dependent on the pol III transcription apparatus and involves the establishment of a discontinuity in arrayed nucleosomes that can interfere with heterochromatin spreading. Histone acetylation is also involved41. The histone acetylation and strong nucleosome depletion we observe at the TSS of active human tRNA genes might produce similar effects. Barrier function has also been ascribed to a B2 gene within the mouse *Growth Hormone* locus, which buffers against encroachment by facultative heterochromatin38. B2 is a pol III-transcribed family of short interspersed elements (SINEs) that are derived from tRNA genes44. B2 SINEs can be bound by USF45, a factor shown to mediate barrier function46. Chromatin modifying enzymes recruited by USF promote H3K4 methylation and acetylation of histone H3, which were proposed to counteract the propagation of heterochromatin46. It is therefore noteworthy that we have found such marks to be a general feature of active pol III-transcribed genes, suggesting that barrier activity may be a widespread property of such loci.

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Another boundary function is to insulate sites against the action of distal enhancers38. A pol III-transcribed SINE belonging to the Alu family has been shown to display such insulator activity in transgenic mice47. The promoter decoy or mimicry model48 posits that insulators recruit components of the transcription machinery that trap enhancers in unproductive interactions. This is strongly reminiscent of the frequent presence of pol II and associated factors that we have discovered at pol III-transcribed genes, perhaps reflecting insulator activity at these sites. Our data therefore raise the possibility that pol III transcription units may play a much larger role than previously envisaged in organizing the human genome into discrete functional domains.

Database accession numbers

ChIP-Seq data have been deposited to NCBI GEO database (GSE20072).

METHODS

ChIP and ChIP-Seq

ChIP assays were performed as previously described33. The antibodies used were described in Supplementary Table 1. ChIP-Seq libraries were created as described previously and sequenced on Illumina Genome Analyzer II10. Basic data analysis was performed using standard Illumina pipeline. Advanced computational analysis is described in Supplementary Methods section. Tag numbers are given in Supplementary Table 1.

ChIP-PCR analyses were performed with the following primer pairs:

5S: Reverse: 5′-GGCATACCACCCTGAACGC-3′

Forward: 5′-CAGCACCCGGTATTCCCAGG-3′

U1: Reverse:5′-CACGAAGGAGTTCCCGTG-3′

Forward: 5′-CCCTGCCAGGTAAGTATG-3′

tRNALeu14: Reverse: 5′-CTCCTATAACGTGTGTGGTAGTGTG-3′

Forward: 5′-ACTGGCAGT GGTGGGATTC-3′

tRNALeu14 upstream: Reverse: 5′-CGATCCAACAGCAAGGAGTT-3′

Forward: 5′-AGCGTCAATGTCTTTGGAAG-3′

tRNALeu14 downstream: Reverse: 5′-CCTGCCTA GACCCTGTTTTG-3′

Forward: 5′-CCGCTGAGTTTTTGCTTTCT-3′

ApoE: Reverse: 5′-CTGTCTCAACCGCTTGCTC-3′

ApoE: Forward: 5′-GGATCCTTGAGTCCTACTCAGC-3′

(These primer sequences are in chromosome 19 and amplify at the very begining of *ApoE gene*.)

Gene Desert: Reverse: 5′-GGTTGGCCAGGTACATGTTT-3′

Gene Desert: Forward: 5′-CATCCCTGGACTGATTGTCA-3′

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(These primer sequences are in chromosome 2: 22646488-22646687 and the nearest expressed region in a human expressed sequence tag (EST)(DA697576) that is 46kb away and the nearest mRNA (AK090620) is 700kb away.)

PCR products were analyzed on 5% native polyacrylamide gels stained with SybrGreen (MolecularProbes, Invitrogen). Serial dilutions of input chromatin were used to ensure PCRs were within a linear range.

RNA-Seq analysis of total RNAs

Total RNAs were prepared using miRNeasy kit from Qiagen. rRNA was depleted from total RNA by hybridization to biotinylated oligonucleotides using Invitrogen Ribominus kit. The resulting RNAs were reverse-transcribed using random primers, converted to double-stranded cDNAs, sonicated and ligated to Solexa adaptors for sequencing as previously described35. Sequenced reads were mapped to the genome in “unique reads only” manner. Because many pol III genes exist as multiple copies located in different areas of the genome, absence of tags mapped to specific genes may be either due to absence of expression or due to failure to map tags uniquely.

Alpha-amanitin inhibition of Polymerase activity

Jurkat cells were plated at 1×10^6 cells/ml in RPMI medium and treated with alpha-amanitine oleate (Sigma) at 250 nM or 1000 nM or with DMSO (control). After 4 hours RNAs were prepared using Qiagen miRNeasy kit, reverse transcribed using random primers and analyzed by qPCR using primers obtained from ABI (18S, ACTB, GAPDH, IRF2) or described previously36–37 (5S, U6, pre-tRNAs).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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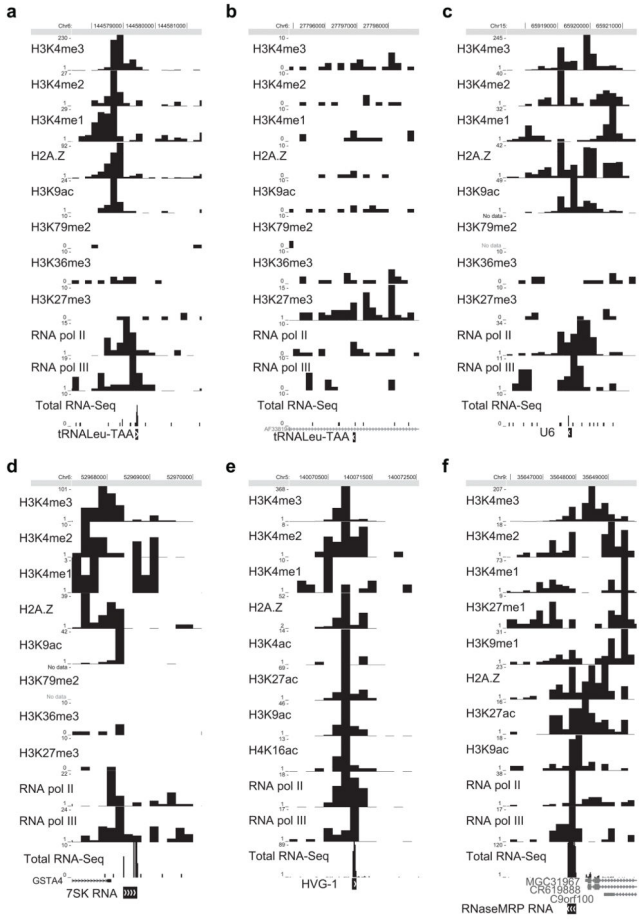

Figure 1.

Chromatin environment of pol III genes. (a,b) Expressed and silent copies of tRNA—Leu-TAA, (c) U6 RNA, (d) 7SK RNA, (e) HVG1 RNA, (f) RNase MRP RNA. The positions of the non-coding RNA genes are indicated at the bottom of each panel. Bars show number of ChIP-Seq tags in 200bp windows and RNA-Seq tags in 20bp windows.

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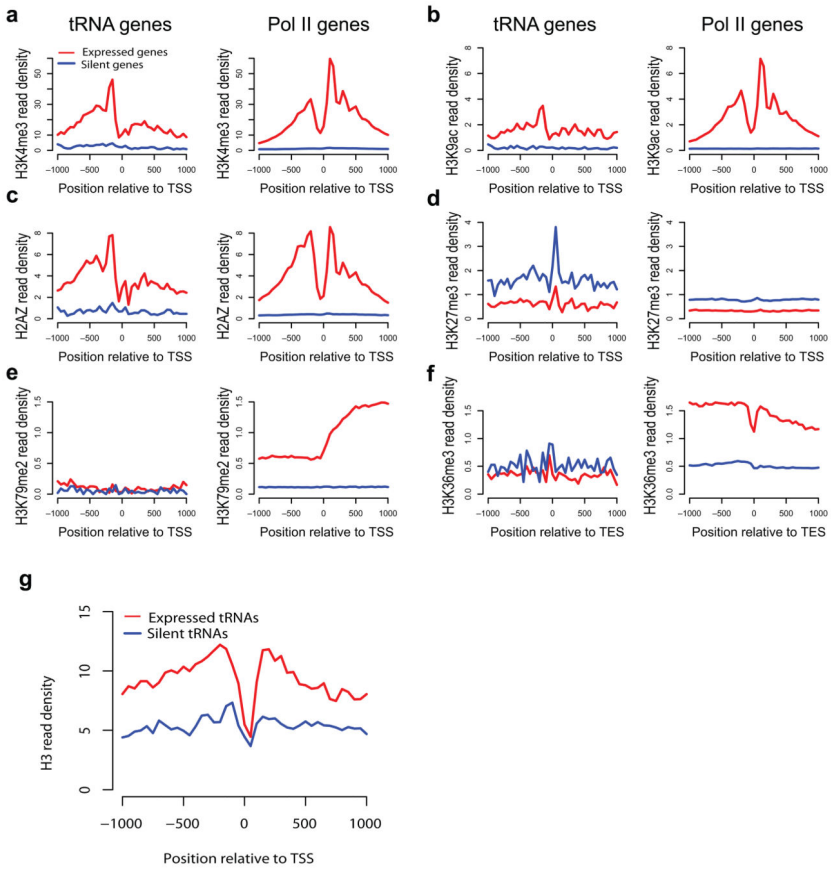

Figure 2.

Pol III target genes are associated with both similar and distinct chromatin features to pol II target genes. Average ChIP-Seq read density (reads per 100 bp) profiles at tRNA (left) and pol II (right) genes are plotted for (a) H3K4me3, (b) H3K9ac, (c) H2A.Z, (d) H3K27me3, (e)H3K79me2, (f) H3K36me3. Genes were imperfectly classified as expressed or silent based on presence of pol III for tRNA genes or pol II for pol II genes. Area surrounding TSS (TES for H3K36me3)- transcription start (end) site is shown. Profiles for other modifications are shown in Supplementary Fig. 1. (g) Histone H3 ChIP-Seq read densities surrounding the TSSs of expressed (red) and silent (blue) tRNA genes.

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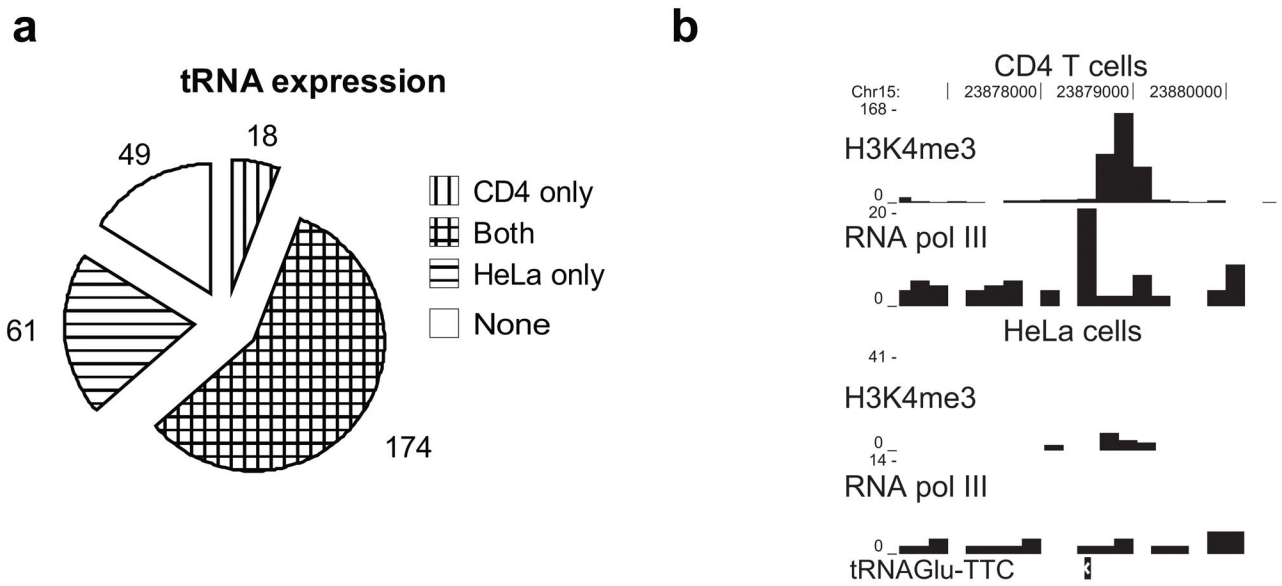

Figure 3.

Tissue specific expression of tRNA genes. (a) Pie chart shows number of tRNAs expressed in HeLa cells, $CD4^+$ T cells, both or neither. (b) Chromatin environment of tRNA—Glu-TTC in $CD4^+$ T and HeLa cells.

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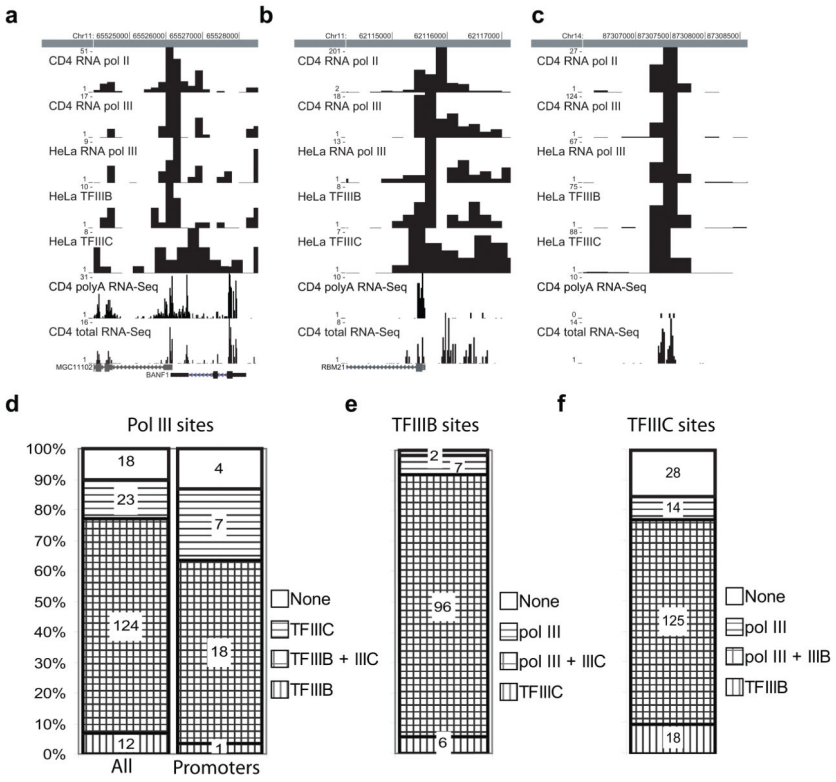

Figure 4.

Novel pol III binding sites. (a,b,c) Novel pol III sites were found in the vicinity of known pol II promoters (a,b) as well as in gene free areas. Presence of total RNA-Seq signals suggests that the binding of polymerase at these loci is productive. Absence of corresponding polyA RNA-seq signal in b and c suggests that these loci are transcribed by pol III rather than pol II. (d) TFIIIB and TFIIIC can be found at the majority of the novel pol III loci.

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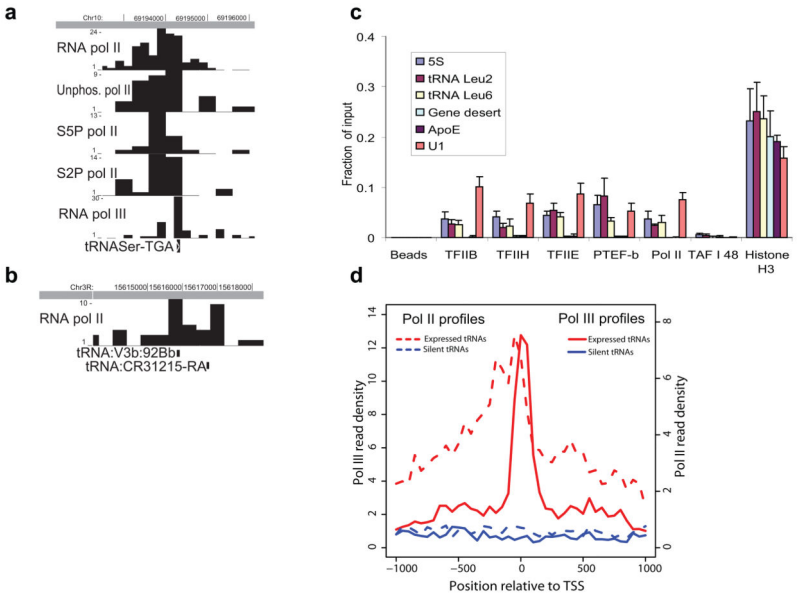

Figure 5.

Pol II is present at pol III-transcribed genes. (a) Various pol II isoforms are present at tRNASer-TGA in human CD4^{+} Tcells, (b) pol II is present at two tRNAs in Drosophila S2 cells. (c) Recruitment of pol II and its factors to various Pol III-transcribed RNA genes. (d) ChIP-Seq tag density profiles of pol II and III in the vicinity of silent and expressed tRNA genes.

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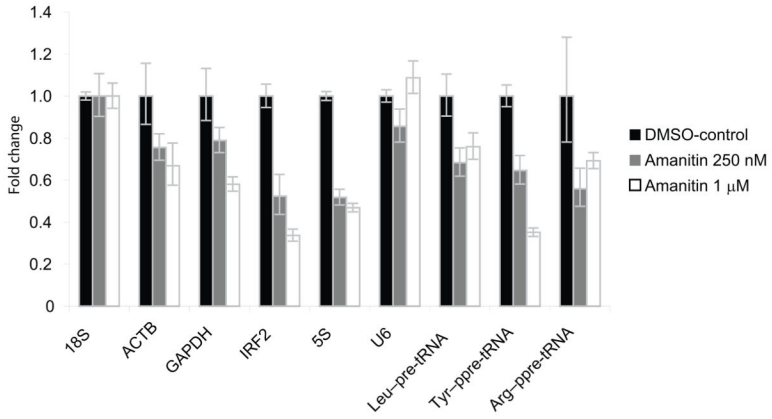

Figure 6.

Levels of 5S rRNA and pre-tRNAs are decreased by 4 hour treatment with alpha-amanitin oleate. qPCR results show that levels of both pol II (ACTB, GAPDH and IRF2) and some pol III genes (5S rRNA and pre-tRNAs) are decreased as a result of short term treatment with pol II inhibitor alpha-amanitin oleate. Mean +/−SD are shown, n=3.