

Selective repression of SINE transcription by RNA polymerase III

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A million copies of the Alu short interspersed nuclear element (SINE) are scattered throughout the human genome, providing ~11% of our total DNA. SINEs spread by retrotransposition, using a transcript generated by RNA polymerase (pol) III from an internal promoter. Levels of these pol III-dependent Alu transcripts are far lower than might be expected from the abundance of the template. This was believed to reflect transcriptional suppression through DNA methylation, denying pol III access to most SINEs through chromatin-mediated effects. Contrary to expectations, our recent study found no evidence that methylation of SINE DNA reduces its occupancy or expression by pol III. However, histone H3 associated with SINEs is prominently methylated on lysine 9, a mark that correlates with transcriptional silencing. The SUV39 methyltransferases that deposit this mark can be found at many SINEs. Furthermore, a selective inhibitor of SUV39 stimulates pol III recruitment to these loci, as well as SINE expression. These data suggest that methylation of histone H3 rather than DNA may mediate repression of SINE transcription by pol III, at least under the conditions we studied.

SINE Expression Can Have Detrimental Consequences and is Subject to Repression

SINEs are retrotransposons that evolved from transcripts made by pol III, most commonly tRNAs.¹ For example,

mouse B2 SINEs evolved from a tRNA,² whereas human Alu and mouse B1 SINEs evolved from the 7SL RNA scaffold of the signal recognition particle.³ The principle pol III promoter elements of tRNA and 7SL genes are located within the transcribed region and are therefore propagated during retrotransposition, a feature that contributed substantially to the prolific spread of SINEs throughout mammalian genomes. Insertion of retrotransposed Alu SINEs has been implicated in many cases of human disease, a problem exacerbated by their concentration near protein-coding genes.^{4,5} Indeed, Alus are responsible for the majority of documented disease cases attributed to the insertion of retrotransposons.⁶ Comparison of 44 human genomes revealed an average of 791 Alu insertions in each that are not found in the reference assembly,⁷ that serves as a representative standard. The number of Alu insertions increases in cancer cells,⁷ perhaps reflecting elevated pol III activity.⁸ SINEs also provide hotspots for recombination between non-allelic loci, undermining genomic stability.^{5,9} In addition, overexpression of SINE transcripts can be cytotoxic and cause macular degeneration.¹⁰ Protective mechanisms have evolved to limit these deleterious effects. One such mechanism is transcriptional suppression and it was estimated that ~99% of potentially active Alus may be subject to chromatin-mediated silencing.¹¹ Genome-wide analyses suggest that only ~0.1% of Alu loci are transcribed or occupied by pol III in the cell lines investigated.^{12–14} Other studies reported even lower pol III occupancy,^{15–17} but this may reflect technical differences, such as the

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choice of statistical threshold. Nevertheless, all agree that there is powerful suppression and the favored model for this has been transcriptional inhibition by DNA methylation.

DNA Methylation at SINEs

SINE DNA is heavily methylated in mouse and human cells.¹⁸⁻²⁰ That this contributes to transcriptional repression was suggested by increased SINE expression induced by 5-azacytidine, an inhibitor of DNA methylation.²¹ However, the cells in these experiments had been treated for 8 days, which is more than long enough for indirect secondary effects. When we analyzed cells after 16 or 72 hrs exposure to 5-azacytidine, SINE expression was unchanged, despite robust induction of Apo-E, a pol II-transcribed gene shown previously to be silenced by DNA methylation.¹⁴ We confirmed that SINE DNA is demethylated in cells treated for 72 hrs with 5-azacytidine, without transcriptional induction.¹⁴ Similarly, SINE expression is unaltered in fibroblasts from mice with genetic knockout of Dnmt1, despite a 20-fold reduction in DNA methylation relative to wild-type.¹⁴ One possibility is that cells compensate for elevated SINE transcription by increasing transcript degradation, so that overall expression is unchanged. However, we saw no change in SINE occupancy by pol III or its associated factors TFIIB and TFIIC in Dnmt1 knockout or 5-azacytidine-treated cells.¹⁴ This argues strongly against the long-standing model that DNA methylation is responsible for denying accessibility of SINEs to pol III. Indeed, bisulphite sequencing after chromatin immunoprecipitation (ChIP-BS-Seq) of pol III-bound Alu DNA revealed no decrease in methylation relative to input genomic DNA.¹⁴ This proved directly that pol III engages methylated Alu DNA in vivo. A possibility remains that template methylation is unfavourable for passage of pol III along the DNA, but we found no evidence to support this conjecture. The data do, however, argue strongly against models in which SINE accessibility to the transcription apparatus is suppressed by DNA methylation.

The opposite conclusion has been drawn in several previous studies, where transcription by pol III was found to be inhibited when the template DNA was methylated.^{19,22,23} Stimulation of transcription by Alu methylation has also been reported.²⁴ A salient difference between our work and these contradictory studies was that the earlier analyses used plasmid DNA either in vitro or in transfected cells. This important difference might account, at least in part, for the discrepancies; perhaps pol III becomes more sensitive to DNA methylation when asked to perform outside the context of chromosomal DNA. It is also likely that transcriptional sensitivity to DNA methylation varies according to circumstances, even in the context of physiological chromatin.

MeCP2 at SINEs

In keeping with their strong methylation, ChIP assays demonstrated that SINEs are bound in vivo by proteins that recognize methylated DNA, such as MeCP2.^{14,25} MeCP2 interacts with histone deacetylases (HDACs)²⁶ and has long been regarded as a transcriptional repressor.²⁷ We had expected MeCP2 to have an inhibitory effect on SINE transcription, as active pol III promoters have acetylated histones^{16,28-31} and the HDAC inhibitor trichostatin A stimulates both pol III recruitment and SINE expression in vivo.^{28,32} However, HDACs are retained at SINEs in Dnmt1-knockout cells, where DNA is predominantly unmethylated, albeit at reduced levels.³³ Sequential ChIP revealed that MeCP2 occupies SINEs simultaneously with pol III. Furthermore, release of MeCP2 following 5-azacytidine treatment was not accompanied by discernible effects on pol III binding or activity.¹⁴ Effects of MeCP2 on histone acetylation, chromatin structure and gene expression are seen predominantly in neurons, where it is far more abundant than in other cell types.³⁴ This may explain its lack of apparent effect in the HeLa, ES cells and fibroblasts used in our study. Furthermore, repression by MeCP2 was recently reported to be proportional to gene length, it having minimal effect on genes shorter than

~100kb;³⁵ with lengths of ~280bp or less, SINEs fall well below this threshold.

H3K9 Methylation at SINEs

Trimethylation of histone H3 on lysine 9 (H3K9me3) is a mark that is highly enriched at inactive tRNA genes, relative to genes that are pol III-occupied.^{16,29} H3K9me3 is also enriched at SINEs, even in the absence of DNA methylation.¹⁴ One of the enzymes that trimethylates H3K9 is SUV39H1, a methyltransferase that we detected at SINEs.¹⁴ SUV39H1 associates with HDACs.³⁶ Furthermore, SUV39H1 and H3K9me3 together provide binding sites for HP1, a heterochromatin-associated protein that mediates transcriptional repression³⁷ and was found at the same SINEs as SUV39H1.¹⁴ Significant decreases in H3K9me3, along with increased pol III occupancy and elevated SINE expression, followed treatment of cells with the fungal mycotoxin chaetocin,¹⁴ a selective inhibitor of SUV39H1 and related histone methyltransferases.³⁸ This is consistent with a previous report that SINE expression can be stimulated by a dominant negative version of Suv39h.³⁹ These data suggest that SUV39H1 contributes to the suppression of SINE transcription. Its involvement is likely to be substantial, as the increased pol III recruitment and expression were detected using consensus primer sets that recognize hundreds of members of the Alu, B1 and B2 SINE families.¹⁴ However, neither SUV39H1 nor HP1 were detected at a particular Alu on chromosome 6, despite enrichment for H3K9me3; in contrast to the other SINEs examined, this Alu showed no change in H3K9me3 or pol III binding following chaetocin treatment. Thus, other histone methyltransferases are also likely to modify H3K9 at some SINEs, presumably depending on local environment.

Although there is no overlap between H3K9me3 and pol III binding in HeLa cells, there is a 24% overlap in human H1 ES cells.³¹ H3K9me3 may be less refractory to pol III recruitment in this context, because HP1 does not associate stably with chromatin in undifferentiated ES cells.⁴⁰ This observation provides support

for the contention that effects of chromatin modification vary according to context. Indeed, SINE expression varies substantially between tissues, with embryos showing notably higher levels of SINE-initiated transcripts.⁴¹ Responses to stimuli also depend on cell type. For example, acute (30 min) restraint of rats is a stress that stimulates SINE expression in most tissues analyzed, including frontal cortex and cerebellum, but has the opposite effect in the hippocampus, where a ~5-fold decrease occurs.⁴² It is intriguing that this selective response correlates with induction of Suv39h1 and a localized increase in H3K9me3 at SINEs in the stressed hippocampus.⁴²

As stated above, Dnmt1 knockout has minimal effect on SINE expression in fibroblasts, despite the loss of DNA methylation.¹⁴ However, we found that SINE induction by chaetocin is substantially enhanced in the absence of Dnmt1.³³ This suggests the possibility that DNA methylation can assume importance for SINE repression under circumstances where SUV39H1 is inactive, potentially providing a back-up mechanism to protect against derepression. Further work will be needed to test this model, as indirect effects could also explain the increased SINE response to chaetocin in the absence of Dnmt1.

SINEs are Inefficient at Recruiting Pol III

The primate Alu family and the rodent B1 SINE family evolved from 7SL RNA, which provides the essential RNA scaffold for the signal recognition particle.³ 7SL genes are highly active and every HeLa cell is estimated to carry ~10⁶ transcripts from the 3 7SL loci per human genome.²¹ In striking contrast, the same study estimated that Alu transcript abundance is only ~10³ transcripts per HeLa cell, despite the ~10⁶ templates dispersed across our genomes.²¹ The transcribed regions of 7SL genes contain an internal promoter sequence that provides the binding site for TFIIC, the transcription factor responsible for recognizing most pol III templates.⁴³⁻⁴⁵ This internal promoter is propagated during retrotransposition,

providing a TFIIC-binding site within each new SINE. Although subsequent mutation has compromised these internal promoters in many SINEs, we detected TFIIC at each of the 5 individual Alu loci examined.¹⁴ Indeed, TFIIC occupancy of these SINEs in HeLa cells is comparable to that of 7SL, as judged by ChIP-qPCR.¹⁴ When consensus primers were used to amplify ~10³ members of one of the younger Alu subfamilies (PV), TFIIC was detected at levels not far below its occupancy of 7SL genes.¹⁴ In contrast, pol III occupancy assessed in parallel was markedly higher on 7SL than on

Alus amplified with either locus-specific or consensus primers.¹⁴ Inefficient recruitment of pol III was also observed on B1 and B2 SINE families in mouse cells.¹⁴ Quantification of multiple ChIP experiments revealed that the ratio of pol III to TFIIC is significantly lower on Alu, B1 or B2 SINEs than it is on 7SL.¹⁴ Thus, SINEs are accessible to TFIIC but then struggle to recruit pol III. When the H3K9me3 mark was removed in cells exposed to SUV39H1 inhibitor chaetocin, pol III occupancy increased significantly with minimal change in TFIIC binding (Fig. 1). Thus, SUV39H1 appears to act

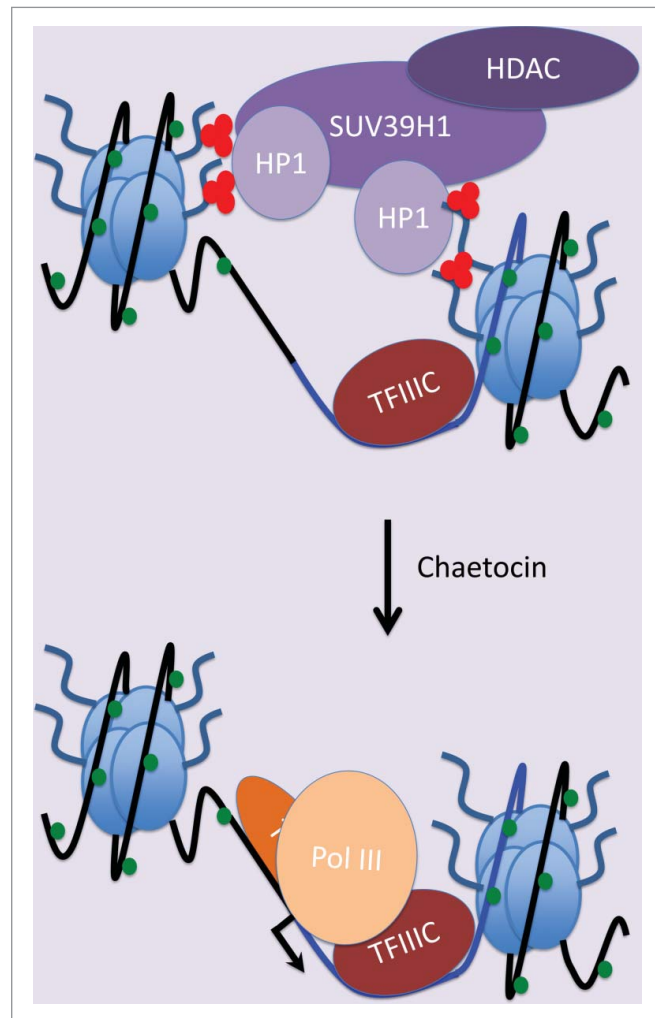


Figure 1. Model of SINE repression by SUV39H1. Lysine 9 of histone H3 is trimethylated in the vicinity of SINEs by SUV39H1, thereby attracting HP1. The resultant repressive chromatin structure impedes pol III recruitment to SINEs, while having much less effect on TFIIC. The SUV39H1 inhibitor chaetocin can alleviate this repression and thereby stimulate transcription of SINEs by pol III. Blue line indicates SINE DNA; black line indicates flanking DNA. Red dots signify trimethylated H3K9. Green dots signify methylated DNA.

at SINEs on a step after template recognition by TFIIC. In contrast, pol III recruitment is highly efficient at 7SL, which shows only background levels of SUV39H1, H3K9me3 and HP1.¹⁴

TFIIC can be regarded as a “pioneer” factor that is good at accessing promoters in chromatin.⁴⁶ Once it has initiated assembly of the transcription complex, recruitment of pol III depends on TFIIB, a factor that is itself recruited by TFIIC to DNA upstream of the transcription

start site.^{47,48} (Fig. 2) For 7SL, the DNA immediately upstream of the start site, where TFIIB binds, has a strong stimulatory effect on transcription.⁴⁹ This upstream sequence from 7SL can also stimulate SINE expression in chimaeric constructs.⁵⁰⁻⁵² However, it is not propagated during retrotransposition and is replaced in SINEs by random DNA flanking the site of insertion, which can influence transcription strongly but is unlikely to be optimal in most cases.¹³ TFIIB can

still be recruited to suboptimal upstream DNA via protein/protein interactions with TFIIC bound to the internal promoter within the SINE, but this is probably less efficient in most cases than for the upstream sequence of 7SL. Indeed, we observed that TFIIB occupancy on SINEs tends to be reduced in comparison to 7SL.¹⁴ However, the relative inefficiency of TFIIB recruitment to SINEs is less marked than for pol III and did not reach statistical significance in our analyses.¹⁴ Thus, a trend toward diminished TFIIB occupancy does not seem sufficient to explain the significant reduction in pol III that is consistently seen at SINEs.

As mentioned above, expression of individual SINEs varies markedly between tissues.^{13,41} Thus, when 7 human cell types were compared, including HeLa and K562, the majority of Alu loci that were expressed in any one cell line appeared to be silent in the other 6.¹³ We detected pol III at ~1,400 Alu SINEs in HeLa cells, but only ~2% of these overlapped with the ~1,000 Alu loci found in a different study to be occupied by pol III in K562 cells.^{12,14} Even within any given cell line, occupancy may vary according to growth conditions.⁵³ Since all Alu SINEs are expected to share the same core machinery (TFIIB, TFIIC, pol III), the variability is most likely caused by factors that bind to flanking regions that are characteristic of each locus and may be tissue-specific and/or sensitive to external parameters. In a particular cell under particular conditions, a tiny fraction of Alu loci may come under the influence of activating factors that promote pol III recruitment. In the absence of such factors, the SINE will probably remain silent, like the majority of its siblings. Such silencing appears to involve histone modification.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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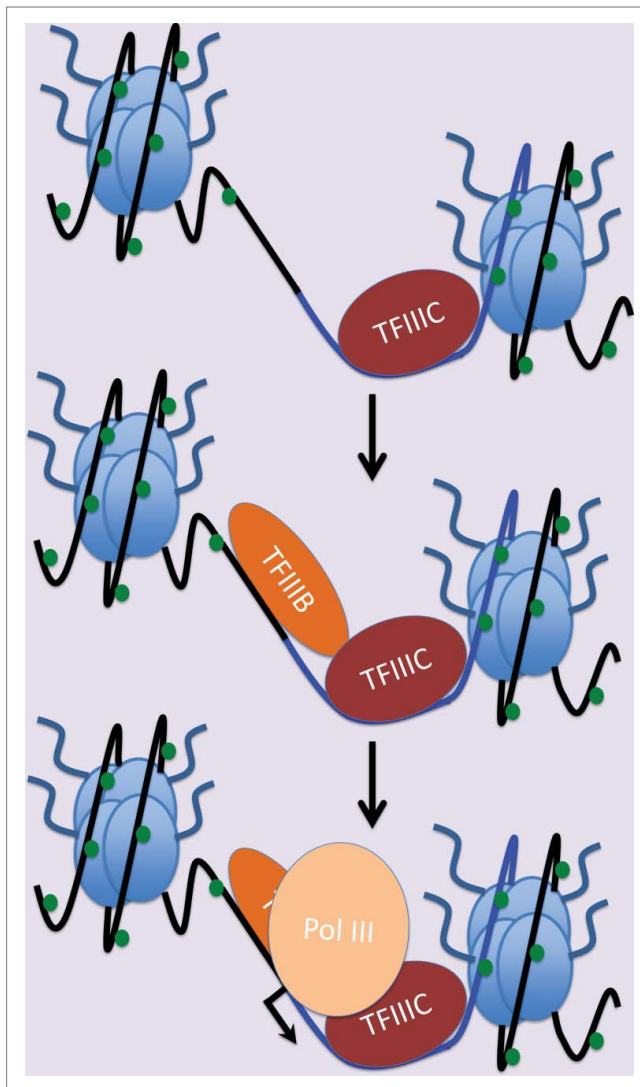


Figure 2. Model of pol III recruitment. Most pol III templates, including 7SL, SINEs and tRNA genes, have promoter sequences within the transcribed region that are recognized by TFIIC. Protein/protein interactions with TFIIC recruit TFIIB and position it on flanking DNA immediately upstream of the transcription start site. TFIIB occupancy can be strongly influenced by upstream DNA sequences (e.g. TATA), but does not depend on a specific DNA recognition motif. Recruitment of pol III is mediated by protein/protein interactions with TFIIB. Blue line indicates SINE DNA; black line indicates flanking DNA. Green dots signify methylated DNA.

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