Enrichment for Histone H3 Lysine 9 Methylation at Alu Repeats in Human Cells*

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The aim of this study was to identify in human cells common targets of histone H3 lysine 9 (H3-Lys9) methylation, a modification that is generally associated with gene silencing. After chromatin immunoprecipitation using an H3-Lys9 methylated antibody, we cloned the recovered DNA and sequenced 47 independent clones. Of these, 38 clones (81%) contained repetitive elements, either short interspersed transposable element (SINE or Alu elements), long terminal repeat (LTR), long interspersed transposable element (LINE), or satellite region (ALR/Alpha) DNA, and three additional clones were near Alu elements. Further characterization of these repetitive elements revealed that 32 clones (68%) were Alu repeats, corresponding to both old Alu (23 clones) and young Alu (9 clones) subfamilies. Association of H3-Lys9 methylation was confirmed by chromatin immunoprecipitation-PCR using conserved Alu primers. In addition, we randomly selected 5 Alu repeats from the recovered clones and confirmed association with H3-Lys9 by PCR using primer sets flanking the Alu elements. Treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine rapidly decreased the level of H3-Lys9 methylation in the Alu elements, suggesting that H3-Lys9 methylation may be related to the suppression of Alu elements through DNA methylation. Thus H3-Lys9 methylation is enriched at human repetitive elements, particularly Alu elements, and may play a role in the suppression of recombination by these elements.

Post-translational modifications of histone tails play a critical role in the assembly of heterochromatin, and accumulating evidence suggests that the pattern of histone modifications specify regulation of gene expression (1, 2). Acetylation of lysine residues on histone H3 and H4 usually leads to the formation of an open chromatin structure, with transcription factors accessible to promoters. Phosphorylation of serine 10 and acetylation of lysine 14 on histone H3 result in gene activation (3). Lys4 methylation on histone H3 localizes to sites of active transcription, and this modification may be stimulatory for transcription (4). The different combinations of histone tail modifications influence transcription by affecting chromatin structure. Recent studies have shown that methylation at Lys4 on histone H3 is a marker of heterochromatin and is specifically associated with inactivation of gene expression (5). H3-Lys9 methylation is enriched on the inactive X chromosome in women (6) and at loci silenced in cancer (7–9). Beyond this, the distribution of H3-Lys9 in human DNA has not been well characterized.

Alu repeats represent the most frequent repetitive element in the human genome (10). Alu elements are ~280 bp in length and consist of two similar, but distinct, monomers linked by an oligo(dA) tract. Presumably, the 1 million Alu elements that are fixed within the human genome represent the fraction of insertions that were neutral or at least tolerable functionally. Alu elements possess an RNA polymerase (pol III) promoter (11). Despite this, the steady state abundance of pol III-directed Alu transcripts is usually very low in tissues and cultured cells indicating transcriptional silencing (12). However, cellular stress can dramatically increase the abundance of human Alu RNA (12), although it is unknown how and how many Alu elements are induced by stress. Accumulating evidence indicates that regulation of Alu expression is determined on many levels, some of which act locally upon individual Alu elements (13, 14). DNA methylation and chromatin configuration might each globally repress transcription of this repetitive sequence family and therefore potentially direct regulation of the Alu stress response.

Here we performed an unbiased approach to clone targets of H3-Lys9 methylation using chromatin immunoprecipitation (ChIP) assay and found that most such clones contain Alu elements. Thus, Alu expression might be crucially regulated by H3-Lys9 methylation and associated chromatin remodeling.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—SW48 cells were grown in L-15 medium (Invitrogen) plus 10% fetal bovine serum in plastic tissue culture plates in a humidified atmosphere containing 5% CO2 at 37 °C. RKO cells were grown in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) plus 10% fetal bovine serum (Intergen). The cells were grown to a density of 5.0 × 106 to 3.0 × 106 cells per dish before being harvested for cross-linking experiments. Cell lines were obtained from the American Type Culture Collection.

5-Aza-dC Treatment of Cells—Cells were split 12–24 h before treatment. Cells were then treated with either 5-aza-dC, 5 μM (Sigma), or phosphate-buffered saline for 72 h. Media containing 5-aza-dC or phosphate-buffered saline was changed every 24 h.

Chromatin Immunoprecipitation—The protocols for ChIP have been described previously (15). Briefly, SW48 and RKO cells are treated with 1% formaldehyde for 8 min to cross-link histones to DNA. After washing by cold phosphate-buffered saline, the cell pellets are resuspended in...
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ChIP with anti-histone H3-K9 methylated antibody

Treat DNA with Mung Bean nuclease

Ligate into blunt-end vector

Sequence randomly selected clones

BLAST/BLAT searches

ChIP-PCR using unique primer sets for confirmation of selected clones

FIG. 1. A schema for the identification of DNA fragments enriched in histone H3-Lys\(^9\) methylation.

| Clone | Size | CG content | Locus | Gene | Accession No. | Location |
|-------|------|------------|-------|------|---------------|----------|
| bp    | %    |            |       |      |               |          |
| 282   | 48   | 17q12      | AluSq,-Sg | AC004099 | 3142-3423    |
| 375   | 48   | 5q33       | AluSp   | AC022411 | 49380-49754  |
| 487   | 40   | 12p13      | AluSx   | AC022424 | 78119-78605  |
| 624   | 45   | 3p26       | AluSx   | AC063953 | 171262-171918 |
| 453   | 51   | 10p24      | AluSq   | AL355512 | 86314-85876  |
| 372   | 48   | 5q33       | AluSp   | AC022411 | 49384-49754  |
| 316   | 56   | Xp22       | AluJo   | AL683870 | 55410-55094  |
| 861   | 39   | Xp22       | AluJo   | AC074035 | 110463-111324 |
| 675   | 38   | 6p21       | AluJo   | AL096865 | 38291-37616  |
| 642   | 41   | 2p11       | AluJb   | AC012484 | 13122-12499  |
| 454   | 42   | 13q12      | AluSg   | AC011731 | 35653-35935  |
| 379   | 48   | 5q33       | AluSp   | AC022411 | 49377-47954  |
| 316   | 50   | 7q11       | AluSc   | AC004927 | 21674-21989  |
| 326   | 37   | 13q31      | AluSc   | AL354892 | 25649-25523  |
| 427   | 43   | 15q22      | AluJo   | AC029756 | 77008-76582  |
| 614   | 41   | 7q21       | AluSg   | AC006045 | 23115-23726  |
| 291   | 34   | 8q24       | AluSx   | AC093328 | 28536-28383  |
| 530   | 45   | 10p11      | AluSx   | AL161651 | 128196-127667 |
| 502   | 46   | 5q13       | AluS/MiLT1a(LTR) | AC010279 | 25771-26272  |
| 448   | 47   | Xq28       | AluSg(MiLR1a(LTR)) | AC107450 | 50718-50270  |
| 744   | 42   | 7p15       | AluSg(LM1A8(LINE)) | AC028866 | 16327-17071  |
| 444   | 22   | 6p21       | AluJol2(LINE) | AL353672 | 4857-5319 |
| 262   | 40   | 11q21      | AluY    | AP003777 | 39404-39865  |
| 150   | 48   | 12p13      | AluY    | AC008012 | 42090-42421  |
| 205   | 36   | 2q33       | AluYe   | AC068121 | 112632-112428 |
| 588   | 45   | 11p15      | AluY    | AC026894 | 36269-36856  |
| 780   | 36   | 2q31       | AluYd2  | AC011238 | 2570-1791   |
| 570   | 42   | 7q11       | AluY    | AC018720 | 12781-11324  |
| 810   | 38   | 8q11       | AluY/LMID2(LINE) | AC064807 | 147277-146471 |
| 396   | 46   | 7q14       | MIR(SINE) | AC005589 | 106331-105567 |
| 865   | 44   | 3q26       | LTR8a(LTR) | AC117459 | 127848-128710 |
| 332   | 48   | 3p24       | MER50v(LTR) | AC094019 | 87736-88067  |
| 687   | 34   | 14q23      | TH1(LTR) | AL161756 | 42351-43036  |
| 860   | 43   | 15q26      | LIME(LINE) | AC015723 | 53257-54117  |
| 273   | 41   | 9p21       | LIP8a(LINE) | AL133261 | 60742-61014  |
| 381   | 51   | 3p24       | L1P8a(LINE) | AC092798 | 45617-45237 |
| 511   | 47   | 4q31       | L2(LINE) | AC104798 | 11983-11473 |
| 429   | 42   | 17q12      | ALR(MiLR1a(LTR)) | X69646 | 642-10790  |
| 769   | 40   | 5p15       | EST, AL118578 | AC091978 | 58983-59661 |
| 583   | 48   | 12q13      | STA76 intron1 | AF178424 | 3316-3898 |
| 450   | 47   | 10p12      | Unknown | AL512753 | 58212-57683  |
| 309   | 51   | 5q14       | Unknown | AC092218 | 86822-87190  |
| 821   | 40   | 8q22       | Unknown* | AC024995 | 93950-94768  |
| 601   | 40   | 5q23       | Unknown* | AC010350 | 37600-38205  |
| 603   | 47   | 6p22       | Unknown* | AL031347 | 146182-146784 |
| 794   | 27   | 7q22       | Unknown | AC007304 | 14254-13479  |
| 614   | 42   | 16p13      | Unknown | U91325 | 32588-33043  |

\(a\) Clones with Alu elements within 200–300 bp.
GGTTTGGCTTTTGAATTG; Alu25-F, ACCTCGTGATCCACCTGCC; Alu25-R, TTTTTTGAAGAACCTCCATACTGCT; Alu27-F, TTTCATTT-TGCTTTCTCACAGATTTT; Alu27-R, GGTCAACAGAGCAAGACTCCGT; Alu43-F, CCCTCCAGTGAACCATCTCTG; Alu43-R, CAAGATCTGCCACTCCACTCC; P21-F, GGTGTCTAGGTGCTCCAGGT; P21-R, GCACTCTCCAGGAGGACACA; MLH1-F, CTTGCTTCTTTTGGGCGT CAT; and MLH1-R, GGCTTGTGTGCCTCTGCTGA; P16-F, AGACAGCCGTTTTACACGCAG; P16-R, CACCGAGAAATCGAAATCACC; GAPDH-F, TCGGTGCGTGCCCAGTTGAACC; GAPDH-R, ATGCGGCTGACTGTCGAACAGGAG.

The PCR products are visualized by agarose or 6% polyacrylamide gel electrophoresis and quantitated by capillary electrophoresis using the Agilent 2100 bioanalyzer (Agilent Technologies). To ensure that PCR amplification was in the linear range, each reaction was initially set up at different dilutions of DNA for varying amplification cycle numbers, and we selected the final PCR conditions accordingly. The assays were done in duplicate.

RESULTS

To identify genes linked to methylated histone H3-Lys9\(^{6}\), we performed ChIP using an anti-Lys\(^{6}\) methylated histone H3 antibody and cloned the immunoprecipitated DNA (strategy described in Fig. 1). Initially, we verified that the library contained methylated H3-Lys9\(^{6}\) enriched DNA fragments by PCR using primers encompassing the promoter regions of P16, hMLH1, P21, and GAPDH. P16 and hMLH1 are silenced, and P21 and GAPDH are expressed in the SW48 cell line (7). In our library, compared with genomic DNA, the former two promoters showed a high degree of enrichment and the latter two promoters showed specific depletion, in accordance with known levels of H3-Lys9\(^{6}\) methylation (data not shown). Having verified the quality of the library, we then randomly selected 47 clones, sequenced them, and characterized the sequences using BLAST (www.ncbi.nlm.nih.gov/) and BLAT (genome.ucsc.edu/) searches.

The results of sequencing are summarized in Table I, and a representative example is shown in Fig. 2. Twenty-four (51\%) of forty-seven clones contained Alu elements only. Five clones had both Alu and long terminal repeats (LTR) or Alu and long interspersed transposable element (LINE). Three additional clones had Alu elements within 200–300 bp of the recovered fragment (clones 43, 44, and 45 had AluSg, -Ye, and -Jb, respectively). Some of the sequences began or ended in the middle of Alu elements, and the range of Alu element length in the recovered clones was 95–282 bp in each clone. Thus, overall, 32 of 47 (68\%) clones were in or within 200 bp of an Alu element.

Considering that Alu elements form about 10\% of the human genome (16), this result indicates marked enrichment for Alu elements in this library, suggesting a high degree of H3-Lys9\(^{6}\) methylation at this repetitive sequence. Twenty-three (72\%) of thirty-two Alu elements belonged to the old Alu subfamily, and...
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In this study we identified Alu elements as major targets for histone H3-Lys9 methylation using chromatin immunoprecipitation. Methylation at Lys9 on histone H3 has been shown to be a marker of heterochromatin and to be associated with inactivation of gene expression (5). In the promoter regions of P16, hMLH1, and MGMT, we recently found increased H3-Lys9 methylation in cells with promoter DNA methylation-associated silencing (7), and others have reported similar findings (8, 9). Thus, H3-Lys9 methylation appears to be a ubiquitous marker of silencing in human cells and likely leads to transcriptional repression by recruitment of a protein complex such as the HP1-associated complex (18).

The repetitive DNA at yeast centromeres is maintained in a transcriptionally silent state by methylation of H3-Lys9 and binding of Swi6 or heterochromatin protein 1 (HP1) to the modified chromatin (19). A recent study of centromere chromatin silencing in the fission yeast, Schizosaccharomyces pombe, demonstrated that silencing of homologous repeats by H3-Lys9 methylation is initially mediated by components of the RNA interference machinery (20). Intriguingly, gene silencing in inactivation of the X chromosome in mammalian female cells involves the expression of Xist RNA, which coats the X chromosome, followed soon after by H3-Lys9 methylation, gene inactivation, and ultimately methylation of DNA (21). The finding of Alu elements as a target of H3-Lys9 methylation raises the possibility that by analogy to yeast silencing of homologous repeats, mechanisms involving RNA interference also operate in the establishment of silenced Alu elements.

Among 32 Alu clones recovered here, the frequency of old Alu elements (23 clones, 72%) and young Alu elements (9 clones, 28%) are consistent with the prevalence of old and young Alu elements in the human genome. In older Alu subfamilies, many of the CpG sites have mutated to TpG or CpA because of deamination of 5-methylcytosine. In contrast, newly retrotransposed Alu elements are CpG-rich and extensively methylated. Our results show that histone H3-Lys9 methylation is characteristic of both old and young Alu subfamilies and suggests that, once established, H3-Lys9 methylation persists over long periods of time, despite eventual inactivation of the Alu elements by promoter mutations.

We also found another repetitive element, LINE, in 7 clones (15%). LINE-1 elements comprise roughly 15% of the human genome and are concentrated in AT-rich regions (16). LINE-1 promoters are also usually methylated at DNA (22), thus providing an explanation for H3-Lys9 methylation. Nevertheless, the frequency of LINE elements in our clones was almost similar to that in the human genome, suggesting that LINE elements are less specifically targeted than Alu elements, al-
though the number of clones analyzed overall in this study is small.

It remains unclear which protein complexes are involved in Alu silencing when these sequences are highly methylated. The SNF2h-NuRD-cohesin complex, which is a chromatin remodeling complex, was recently reported to specifically associate with some Alu elements, accompanied by histone H3-Lys 4 methylation and either histone H3 or H4 acetylation (29). In that study, the binding of the cohesin complex to chromatin around Alu elements could be increased by inhibition of DNA methylation. In our study, treating cells with 5-aza-dC decreased the degree of H3-Lys 9 methylation in all 5 Alu elements tested. It is possible then that the SNF2h-NuRD-cohesin complex counteracts the activity of a silencing complex recruited by histone H3-Lys 9 methylation and that normally reside on Alu elements, possibly as a result of DNA methylation in these regions.

An obvious functional significance of these findings is to provide a molecular mechanism for the known silencing of Alu elements. This silencing is thought to be essential in reducing the mutational load associated with active Alu elements in dividing cells, as has been proposed in the genome defense hypothesis (24). It is of interest, however, that H3-Lys 9 methylation can spread in cis, as seen in fission yeast (25). If similar spreading occurs in human cells, one might observe time-dependent spreading of silencing, a hypothesis consistent with the observations of age-related DNA methylation in normal tissues (26, 27). Furthermore, such Alu element H3-Lys 9 methylation may serve as an initial seed for gene silencing in cancer, as suggested by previous studies (28–30).

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