

Demethylation of host-cell DNA at the site of avian retrovirus integration[☆]

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Abstract

The transcriptional activity of an integrated retroviral copy strongly depends on the adjacent host-cell DNA at the site of integration. Transcribed DNA loci as well as *cis*-acting sequences like enhancers or CpG islands usually permit expression of nearby integrated proviruses. In contrast, proviruses residing close to cellular silencers tend to transcriptional silencing and CpG methylation. Little is known, however, about the influence of provirus integration on the target sequence in the host genome. Here, we report interesting features of a simplified Rous sarcoma virus integrated into a non-transcribed hypermethylated DNA sequence in the Syrian hamster genome. After integration, CpG methylation of this sequence has been lost almost completely and hypomethylated DNA permits proviral transcription and hamster cell transformation by the proviral *v-src* oncogene. This, however, is not a stable state, and non-transformed revertants bearing transcriptionally silenced proviruses segregate with a high rate. The provirus silencing is followed by DNA methylation of both provirus regulatory regions and adjacent cellular sequences. This CpG methylation is very dense and resistant to the demethylation effects of 5-aza-2'-deoxycytidine and/or trichostatin A. Our description exemplifies the capacity of retroviruses/retroviral vectors to overcome, at least transiently, negative position effects of DNA methylation at the site of integration.

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CpG methylation and histone deacetylation are important mechanisms controlling gene expression in vertebrates (see [1] for reference). Retroviral copies or retrovirus-based vectors integrated into host genomes are subjected to the same type of transcriptional control as evidenced by the correlation between hypermethylation of proviral sequences and their transcriptional inactivity [2]. Demethylation of silenced proviruses by molecular cloning leads to the restoration of their transcription and infectivity [3,4]. The level of tran-

scriptional inactivation and CpG methylation patterns of proviral regulatory sequences, in particular long terminal repeats (LTRs), are strongly dependent on the cell type or host-cell differentiation [5], most probably due to the different offer of recruitable cellular factors interacting with a retroviral promoter/enhancer. It has been demonstrated that in the presence of the Tax protein, the methylation suppression of human T-cell leukemia virus type 1 (HTLV-1) LTR is released [6]. In contrast, negatively acting factors are implicated in the inactivation and methylation of the enhancer repeat unit within the Moloney murine leukemia virus (MoMuLV) LTR in embryonic carcinoma cells [7]. DNA methylation might, therefore, be one of the multiple mechanisms of cellular non-permissiveness to retroviruses and a barrier against their spread into foreign species [8].

Despite the recent reports on HIV-1 preferential integration into genes [9,10], the heated discussion about the

[☆] *Abbreviations:* 5-azaC, 5-aza-2'-deoxycytidine; Dnmt, DNA methyltransferase; HEF, hamster embryo fibroblast; HDAC, histone deacetylase; LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; RSV, Rous sarcoma virus; TSA, trichostatin A.

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randomness of retroviral integration into vertebrate host genomes has not been closed so far and many papers evidence both random and preferential integration (see [11] for the review). It is, however, clear that CpG methylation and transcriptional activity of the integrated provirus depend on its chromosomal position [12]. Transcriptionally active Rous sarcoma virus (RSV) proviruses are found close to CpG islands whereas silenced proviruses are not [13]. These findings led to the widely accepted conclusion that proviruses or retrovirus-based vectors are inactivated in most chromosomal positions with the exception of those protected from de novo DNA methylation. Several strategies of anti-methylation protection were tested and proven to maintain long-term and efficient expression of reporter retroviral vectors [14–18], further confirming the importance of the integration site.

Integration of foreign plasmid, bacteriophage or adenovirus-12 DNA into the recipient mammalian genome has been shown to elicit perturbations in CpG methylation and gene transcription in the site of integration [19] and, more interestingly, even in sequences remote from the inserted adenovirus vector or bacteriophage λ [20,21]. Hypomethylation of integration sites [19] as well as increase of methylation [20,21] were documented. The changes in recipient DNA are strongly dependent on the site of foreign DNA insertion [22].

However, little is known about the influence of the integrated retrovirus on the adjacent chromosomal DNA methylation and on the capacity of the provirus to overcome the down-regulating effect of the host-cell genome. To investigate this problem, we have used the well-defined hamster tumor cell line H-19 harboring the single RSV-derived proviral structure LTR, *v-src*, LTR. Transformed H-19 cells transcribe the *v-src* oncogene efficiently but segregate non-transformed revertant clones with a high rate. The morphological reversion of H-19 cells is correlated with the loss of *v-src* mRNA and hypermethylation of proviral sequences [4]. We have studied the CpG methylation of both proviral and adjacent cellular DNA sequences in transformed and revertant H-19 cells by bisulfite treatment and sequencing and compared it with the methylation of the intact integration site in control hamster DNA. We have concluded that the provirus integrates in the hypermethylated site and causes almost complete demethylation. After reversion, however, the CpG methylation turns back to the original level and is maintained even in the presence of DNA methyltransferase (Dnmt) and histone deacetylase (HDAC) inhibitors 5-aza-2'-deoxycytidine (5-azaC) and trichostatin A (TSA), respectively.

Materials and methods

Cells and animals. The origin of the tumor cell line H-19 has been described previously [23]. H-19 cells contain the transforming proviral

structure LTR, *v-src*, LTR, which arose by reverse transcription and integration of RSV *v-src* mRNA [24]. Segregation of non-transformed revertant cell clones from the H-19 cells and isolation of revertant clones H-19r1 and H-19r3 were described previously [4]. Two independent hamster tumor cell lines, H-18 and H-20, that contain RSV proviruses integrated at other genomic loci [23] were used as controls. Hamster embryo fibroblasts (HEFs) were prepared by standard procedures from 10-day-old Syrian hamster (*Mesocricetus auratus*) embryos of the inbred population maintained at the Institute of Molecular Genetics, Prague. H-19 cells, revertants, and HEFs were maintained in the mix of two parts of Ham's F10 medium and one part of Dulbecco's modified Eagle's medium supplemented with 5% calf serum, 5% fetal calf serum, and penicillin/streptomycin in 5% CO₂ atmosphere at 37 °C and passaged twice weekly. DNA of adult hamsters was isolated from kidneys of the pregnant females sacrificed for HEF preparation. HEFs and adult kidney DNA were chosen to match the originally infected hamster cell.

Bisulfite cytosine methylation analysis. DNA samples for bisulfite analysis were prepared and purified using the QIAamp Tissue Columns (Qiagen) according to manufacturer's instructions. Samples of DNA isolated from H-19, H-19r1, H-19r3, and HEF cells or from adult hamster kidney tissue were digested by *Hind*III restriction endonuclease. Bisulfite treatment was performed according to Hájková et al. [25]. Nested or semi-nested PCR of the upper strand was primed with oligonucleotides A, B, C, D, E, and F complementary to the bisulfite-converted sequence of the integration site 5' to the provirus (primer A 5'-AAAGTATTAAGGAGTAGAATTATAGATTTT-3' and primer B 5'-TAAATGTATTTGTATGTATTTGTTTT-3' match nucleotides 452–423 and 293–267 5' to the provirus, respectively), U3 region of the 5' LTR (primer C 5'-TTACATAAACATATTACAAAACACTACAAAAA-3' matches nucleotides 215–186 5' to the transcription start), U3 region of the 3' LTR (primer D 5'-CAGTGAGCTCGTTTTATAAGGAAAGAAAAG-3' matches nucleotides 178–159 5' to the transcription start), and integration site 3' to the provirus (primer E 5'-AACTAC TCCTAAAACCTCCTCCCCT-3' and primer F 5'-CCTTCTCCTC CCAAACCCAAAACAAA-3' match nucleotides 167–193 and 89–116 3' to the 3' LTR, respectively). Primers A, B, and D contain T and primers C, E, and F contain A instead of C in positions complementary to non-methylable C, i.e., C outside CpG dinucleotides. The representation of primer location as well as the size of amplified DNA sequences are given in Fig. 1. PCRs were carried out with ca. 100 ng of bisulfite-treated DNA as follows: 5 min denaturation at 95 °C and 25 cycles of 1 min denaturation at 95 °C, 2 min annealing, and 1 min synthesis at 72 °C. Annealing temperatures were 56 °C for both first A–C and semi-nested B–C PCRs, 58 °C for the first D–E and 59 °C for the semi-nested D–F PCRs, and 57 °C for the first A–E and 56 °C for the nested B–F PCRs. Annealing temperatures for efficient PCR amplification were optimized in advance using an Eppendorf gradient PCR cycler. Final PCR products were cloned into the pGEM-T vector (Promega) according to manufacturer's instructions and sequenced by using universal pUC/M13 forward and reverse primers. Sequencing reactions were incubated in a PCR thermal cycler PTC-225 Tetrad (MJ Research) and analyzed in an ABI PRISM 310 Capillary Genetic Analyzer (Perkin-Elmer Biosystems). Only PCR clones with all Cs outside CpGs converted to Ts were taken into account. At least seven fully converted PCR clones were analyzed from each PCR.

Treatment of cells with 5-azaC and/or TSA. The effect of Dnmt and HDAC inhibitors was tested as described previously [26]. Revertant H-19 cells were plated at the density of 3×10^5 cells/dish. Twenty-four hours later, the culture medium was replaced with medium containing 100 μ M 5-azaC (Sigma) and/or 50 ng/ml TSA (Sigma). After two-day cultivation, the treated cells were harvested for soft agar assay or DNA analysis. Indicated concentrations of 5-azaC and TSA were the highest that had not severely decreased the proliferation of H-19 cells in a pilot experiment.

Soft agar assay. Screening for retransformed cell clones was done by soft agar assay. Cells treated with 5-azaC or TSA were seeded in

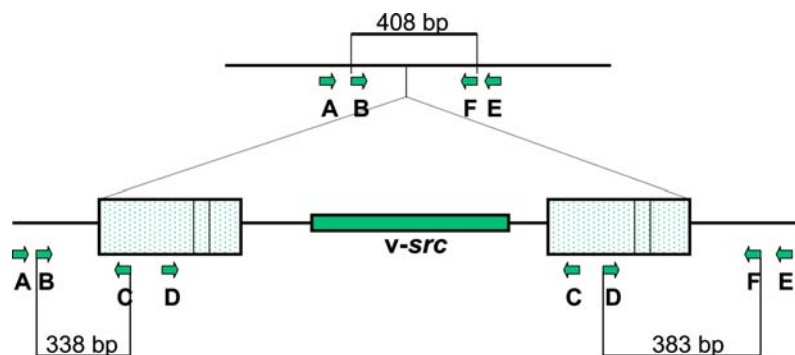


Fig. 1. Schematic representation of the H-19 provirus (LTRs shown as dotted boxes) and localization of primers (arrows) used for semi-nested PCR amplification of the integration site sequences. An intact integration site is depicted on the top. The sizes of amplified DNA fragments are indicated. The lengths of LTRs, *v-src* coding sequence, and amplified DNA fragments are out of scale.

medium supplemented with 0.33% agar over solidified 0.75% agar medium. During 2 weeks of the assay, cultures were refed three times with 2 ml soft agar medium. Both bottom agar and seeding soft agar mediums were supplemented with 100 μ M 5-azaC and/or 50 ng/ml TSA. Refeeding soft agar medium was supplemented with 200 μ M 5-azaC and/or 100 ng/ml TSA to restore concentrations of inhibitors, which are unstable in solution under culture temperature. The growth of colonies in soft agar was assessed after 14 days.

DNA sequence of the integration site. The nucleotide sequence of 1.3 kb region of the integration site covering sequences analyzed here is accessible in GenBank/EMBL Data Bank with No. X95548.

Results

CpG methylation of sequences adjacent to the transcriptionally active provirus

To describe the pattern CpG methylation within the cellular sequences flanking the LTR, *v-src*, LTR provirus in H-19 cells, we have isolated high-molecular DNA from cultivated H-19 cells and exposed it to bisulfite reaction. Using primers A, B, C, and D, E, F, we have amplified the DNA sequences adjacent to the 5' and 3' ends of the provirus, respectively, together with a part of respective LTRs. Sequencing of cloned semi-nested PCR products showed almost unmethylated cellular DNA in the site of integration and weak, randomly scattered methylation of 3' LTR (Figs. 2B and 3B). This finding correlates with our previous observation that the internal H-19 proviral sequences are hypomethylated in transcriptionally active state [4]. Together with the integrated retrovirus, its flanking cellular sequences are hypomethylated as well.

CpG methylation of sequences adjacent to the transcriptionally silenced provirus

Using the same procedure and the same primers, we have analyzed the pattern of CpG methylation within the flanking sequences of transcriptionally silenced proviruses from revertant clones H-19r1 and H-19r3. We have found heavily methylated sequences both 5' and 3' to the

provirus in both H-19r1 and H-19r3 revertant clones (Figs. 2C, D and 3C, D). At the 3' end of the provirus, the LTR sequence was amplified together with the flanking cellular sequence. The CpG methylation of LTR is as dense as that of the adjacent cellular DNA. CpG methylation of internal proviral sequences was described previously [4] and now we show that inactivation of proviral expression correlates with CpG methylation of both proviral and flanking cellular sequences.

CpG methylation of the intact integration site H-19 without integrated provirus

Using nested primers A, B, F, and E we have amplified and analyzed the CpG methylation pattern of the integration site from the non-infected HEFs and from the DNA of a non-infected adult hamster female. We suppose that the original H-19 sarcoma induced in a newborn hamster by RSV arose from a single infected and *v-src*-transformed fibroblast; therefore, the cultivated HEFs should be a relevant control for comparison of the methylation pattern before and after retrovirus integration. Within the 408-bp long sequence of the intact integration site (Fig. 4A) from the HEF genome, we have found dense methylation of CpG dinucleotides with few scattered non-methylated CpGs (Fig. 4B). The same level of CpG methylation was observed in DNA from an adult hamster (Fig. 4C). We conclude that the intact integration site of the provirus H-19 is heavily methylated in the hamster genome. Integration of the provirus almost completely erases this methylation and hypomethylation is maintained as long as the provirus remains transcriptionally active. Concomitant with provirus silencing and methylation of its DNA, flanking sequences revert to their original hypermethylated state.

Resistance of the CpG methylation to the Dnmt and HDAC inhibitors and cell transformation

Previously [4], we had described the lack of retransformation and restoration of *v-src* expression in H-19

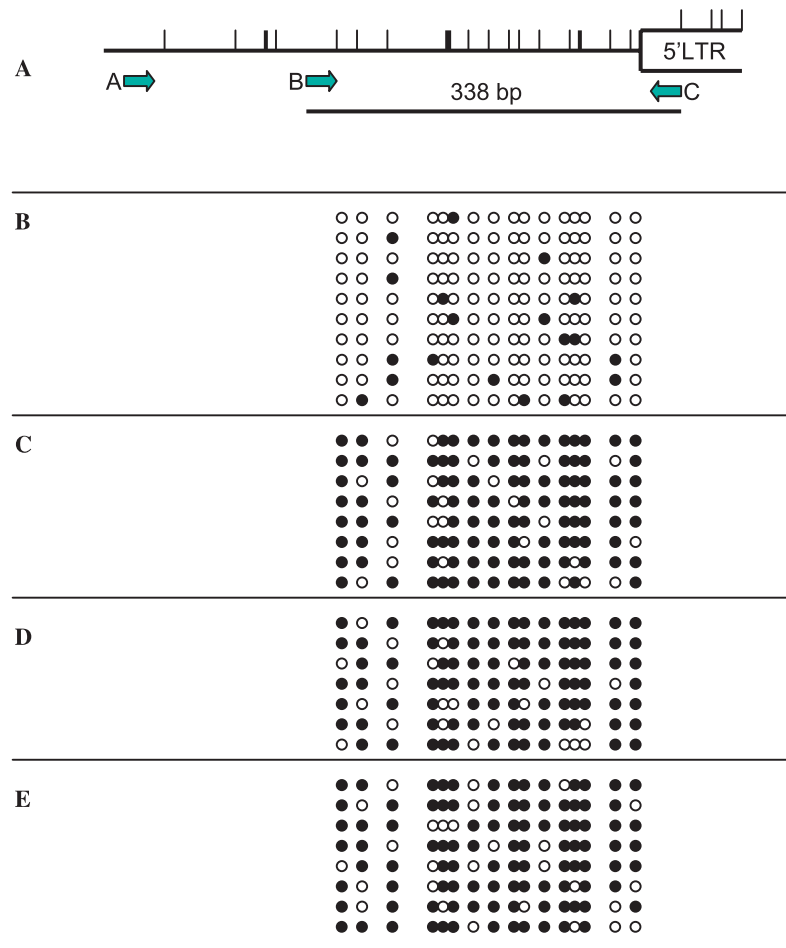


Fig. 2. CpG methylation of the cellular DNA adjacent to the 5'LTR. Localization of CpG dinucleotides (vertical bars) and PCR primers (arrows) within the 5' part of integration site (A). Patterns of CpG methylation within the amplified DNA region from transformed H-19 cells (B), revertant clones H-19r1 (C), and H-19r3 (D), and revertant clone H-19r1 treated with 5-azaC and TSA (E). Methylated CpGs are depicted by filled circles, non-methylated CpGs by empty circles.

revertant clones treated with 5-azaC. To test the effect of inhibitors of DNA methylation and histone deacetylation on the CpG methylation pattern, we have cultivated H-19r1 and H-19r3 cells in the presence of 5-azaC, TSA, or both inhibitors and using semi-nested primers A, B, C and D, E, F analyzed the CpG methylation in the host genome DNA flanking the provirus 5' and 3' to the provirus, respectively. We have not detected any change in the density or pattern of DNA methylation in any of the revertant clones and in any of inhibitor combinations (results of bisulfite sequencing are shown only for H-19r1 in Figs. 2E and 3E). Using nested primers A, B, E, and F, we have analyzed the methylation of the intact integration site in the DNA from HEFs cultivated in the presence of 5-azaC and TSA. Again, we have not detected any significant difference between the methylation of treated and non-treated HEFs (Fig. 4D). To be sure that 5-azaC acts in our experiment as an inhibitor of methylation, we isolated DNA from normal and 5-azaC, TSA-treated revertant cells and compared their *HpaII* digests in agarose gel under UV light. DNA from 5-

azaC, TSA-treated cells provided smaller fragments than did DNA from untreated cells (data not shown).

In parallel, we have tried whether the combinatory effect of azaC and TSA could retransform the H-19 revertant cells by reactivation of the silent provirus. Despite the fact that the concentration of 5-azaC was higher than that in our previous study [4], we have not observed colonies of retransformed cells growing in the semisolid medium (data not shown). This fact further confirms that demethylation of the silent H-19 provirus as well as of its integration site is extremely inefficient.

In order to show that the demethylation of DNA at the site of provirus integration was not simply an effect of cell transformation, we have analyzed the CpG methylation patterns at the H-19 integration site in two independently transformed hamster tumor cell lines, H-18 and H-20. The transforming RSV proviruses reside at different genomic loci in these cell lines and, therefore, the H-19 integration site remains intact here. We have amplified these 408 bp sequences using the nested primers A, B, F, and E and analyzed their CpG meth-

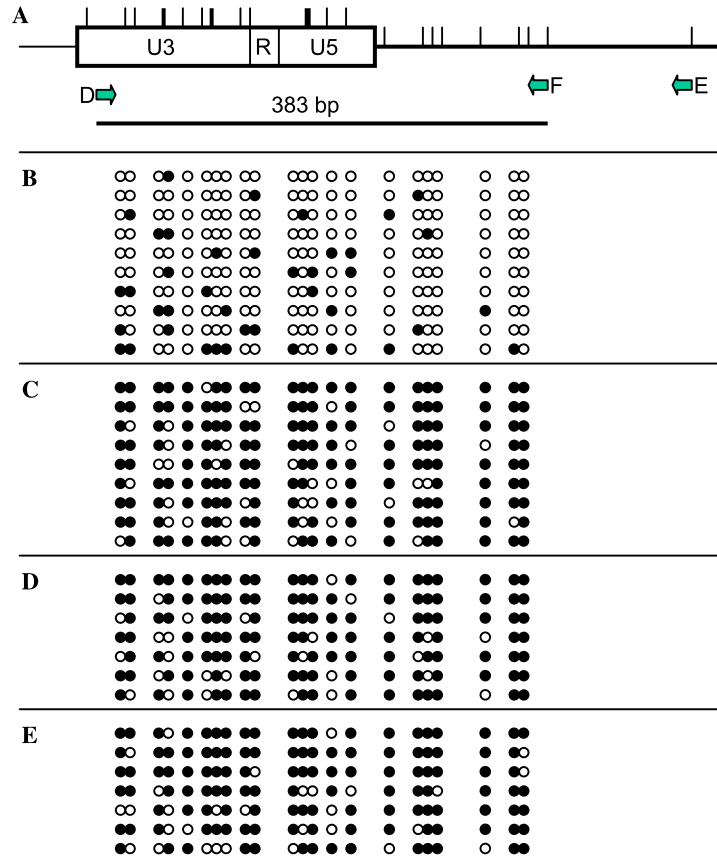


Fig. 3. CpG methylation of the 3'LTR and adjacent cellular DNA. Localization of CpG dinucleotides (vertical bars) and PCR primers (arrows) within the LTR and the 3' part of the integration site (A). Patterns of CpG methylation within the amplified DNA region from transformed H-19 cells (B), revertant clones H-19r1 (C), and H-19r3 (D), and revertant clone H-19r1 treated with 5-azaC and TSA (E). Methylated CpGs are depicted by filled circles, non-methylated CpGs by empty circles.

ylation patterns. We show that these sequences are densely methylated in both H-18 and H-20 cell lines (Figs. 4E and F) and, therefore, the cell transformation alone is not the case of DNA demethylation at this site.

Discussion

The present study demonstrates the case of RSV integration into highly methylated locus in a non-permissive mammalian genome. After provirus integration, this locus almost completely loses CpG methylation and permits provirus expression and cellular transformation. This demethylation, however, is not stable; the original density of CpG methylation is restored with high frequency in revertants segregating from the transformed cell line. Together with the adjacent cellular sequences, the proviral DNA is de novo methylated and the provirus is irreversibly transcriptionally silenced. Interestingly, methylation of the integration locus is resistant to inhibitors of Dnmts and HDACs.

Interaction of a provirus with its flanking host DNA sequences at the level of CpG methylation and tran-

scription has not been resolved yet. There are many data about the preference of provirus integration, little is, however, known whether the integrated provirus exerts some influence on the transcription and methylation status of the integration site. Methylation of three sequences flanking MoMLV proviruses was described and compared with the methylation of relevant loci from non-infected mice strain. While the intact genomic loci are non-methylated the same sequences flanking the proviruses are methylated in correspondence with the transcriptional silence of the proviruses [27]. Because this study was done using established mouse strains bearing unique and silent proviruses, it could not be excluded that immediately after integration these proviruses were transcribed. Our experiments describe both transcriptionally active and inactive states of the same provirus in a successive order of events after the infection of a host cell. Furthermore, we have used the bisulfite sequencing analysis describing methylation at each CpG dinucleotide of the studied DNA sequence.

Retrovirus integration into a methylated genomic region, as described here, is not quite unique. Although usually the transcriptionally active and decondensed

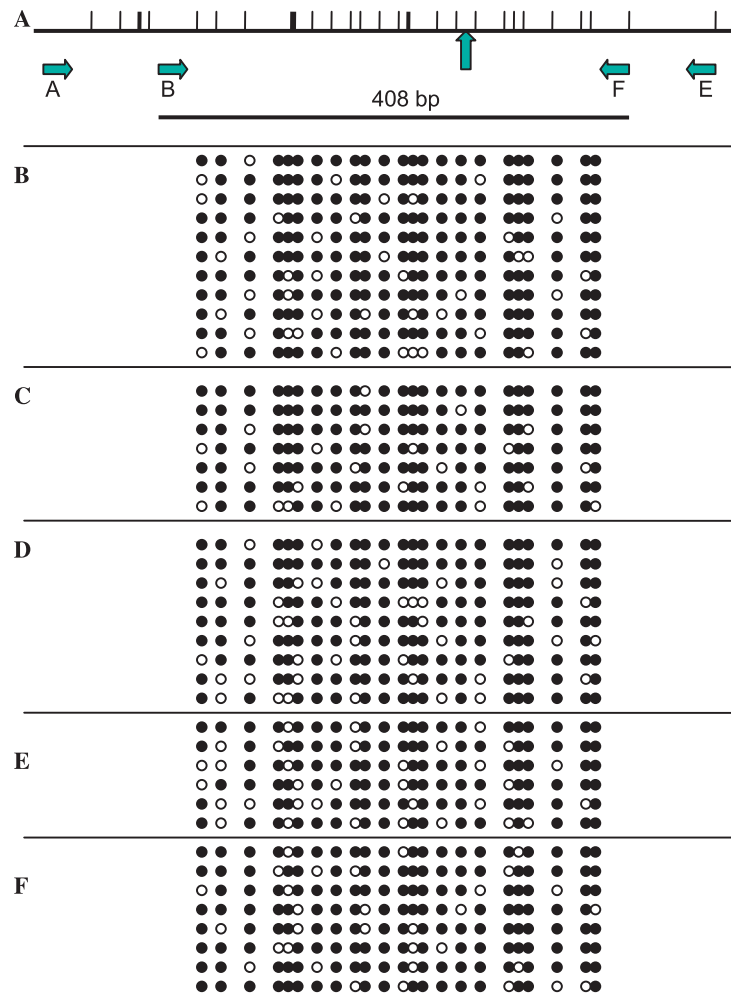


Fig. 4. CpG methylation of the intact site of H-19 provirus integration. Localization of CpG dinucleotides (vertical bars) and PCR primers (horizontal arrows) close to the integration site (vertical arrow) without provirus (A). Patterns of CpG methylation within the amplified DNA region from HEFs (B), adult hamster kidney (C), HEFs treated with 5-azaC and TSA (D), H-18 tumor cell line (E), and H-20 tumor cell line (F). Methylated CpGs are depicted by filled circles, non-methylated CpGs by empty circles.

chromatin is supposed to represent a preferred target of retroviral integrase, there are several studies describing integration into methylated sequence. For example, Kitamura et al. [28] showed by in vitro integrase assay a strong bias for integration of avian leukemia virus pre-integration complexes into methylated CpG runs on a target plasmid. This corresponds with our finding that the sequence of 230 bp immediately upstream to the provirus represents a short CpG island. Although not detected by the presence of CpG island-specific restriction sites in our previous study [29], this sequence with 59.6% GC, 13 CpG, and 15 GpC dinucleotides (ratio CpG/GpC = 0.87), ratio of observed/expected CpGs of 0.64, and its length more than 200 bp meets all arbitrary criteria set for CpG islands [30]. One possible explanation of the observed integration preference for methylated CpG regions may be the methylation-induced distortion of the local DNA structure from B- to Z-DNA [28,31].

The apparent lack of CpG demethylation in the provirus flanking sequences is consistent with our pre-

vious failure to retransform H-19 revertant cells and restore *v-src* expression [4]. Lack of the demethylating effect of 5-azaC or even hypermethylation of transgene sequences by 5-azaC was described earlier [32–34]. Such resistance to 5-azaC also excludes the fact that the local demethylation observed in transformed H-19 cells can be attributed to the global changes of DNA methylation occurring in a cancer cell [35,36]. This is further corroborated by the finding of methylated H-19 integration locus in two hamster tumour cell lines transformed independently by RSV provirus integration at other genomic positions. If the strong resistance to demethylation or specific and continuous de novo methylation of H-19 flanking sequences has any relation to successful and regular integration of highly simplified and expressed provirus remains a matter of speculation.

It should be stressed that our results represent only one event of retrovirus integration and that this might be rather peculiar case of avian retrovirus artificially introduced into the mammalian genome. Such hetero-

transmission might be strongly affected by the RSV LTR sensitivity to CpG methylation [37] and rare features of the integration site. Nevertheless, in the case of frequent retroviral integration into methylated regions of the host genome, the proviral capacity to demethylate its flanking sequences could be important for the outcome of infection. Although not entirely unexpected, this interplay between provirus and host DNA sequences is for the first time described in a dynamic and accurate way in our study.

Acknowledgments

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Note added in proof. Recently, we have found a highly significant homology of the hamster H-19 integration site to a mouse chromosome 1 locus containing the gene for collagen alpha 1 type XXIV (Accession number for partial mouse mRNA sequence AY243578). According to a homology with the corresponding full length human collagen gene (Accession number AY244357), the provirus is integrated in the 5' end of the gene in opposite orientation.

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