Differential inheritance modes of DNA methylation between euchromatic and heterochromatic DNA sequences in ageing fetal bovine fibroblasts

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Abstract To elucidate overall changes in DNA methylation occurring by inappropriate epigenetic control during ageing, we compared fetal bovine fibroblasts and their aged neomycin-resistant versions using bisulfite–PCR technology. Reduction in DNA methylation was observed in euchromatic repeats (18S-*rRNA*/*art2*) and promoter regions of single-copy genes (the cytokeratin/β-lactoglobulin/interleukin-13 genes). Contrastingly, a stable maintenance of DNA methylation was revealed in various heterochromatic sequences (satellite I/II/alphoid and *Bov-B*). The differential inheritance mode of DNA methylation was confirmed through the analysis of individual neomycin-resistant clones. These global, multi-locus analyses provide evidence on the tendency of differential epigenetic modification between genomic DNA regions during ageing. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ageing; Methylation; Epigenetic modification; Euchromatin; Heterochromatin

1. Introduction

The pattern of DNA methylation in any given cell occurs as a consequence of the dynamic process of methylation and demethylation [1] and, once established, the methylation patterns are stably maintained without significant change from one cell generation to the next [2]. Exceptions to this stability in maintenance of methylation through the generations, however, are commonly observed from ageing cells and neoplastic cells (reviewed in [3,4]). One of the most frequently observed methylation changes in both ageing and cancer cells is hypomethylation of overall genomic DNA.

Now, the list of genomic loci demethylated during ageing is increasing. Nevertheless, due to the use of different target experimental systems (animal species/organs/tissues/cultured cells) and of a limited number of genomic loci in each experiment, it is still difficult, or even confusing, to make an outline of overall senescence-related methylation changes from such heterogeneous findings. Here, using a bisulfite–PCR technology, we examined many genomic loci of primary cultured bovine fibroblasts and their aged counterparts. We believe that this study would provide an opportunity to arrange the overall pattern of methylation changes at certain time points of the ageing process and thus to put the varied findings on age-related methylation changes in order.

2. Materials and methods

Fetal bovine fibroblasts were isolated from the carcass of a day 40 male fetus and cultured as described before [5]. Fetal fibroblasts were counted at near confluence and subcultured fourfold, with each successive passage constituting two population doublings (PDL). Cells at 6 PDL were transfected with 2 μg of plasmid pSV2neo (Clontech) using Transfectamine (Life Technology) according to the supplier's recommendation. After 14–18 days of G418 selection (600 μg/ml, Life Technology), rapidly growing colonies were individually isolated using a cloning cylinder (Sigma), and transferred serially to 96-well, 48-well, 6-well, and finally to ∅100-mm dishes. These clones consisting of cells at approximately 33 PDL showed negligible growth.

Cells in a confluent state were solubilized in lysis buffer containing 200 μg/ml proteinase K and then genomic DNA was isolated as previously described [6] and treated with bisulfite as described previously [7]. For the amplification of the target region, PCR was performed three times, each time with 100 ng of bisulfite-treated genomic DNA. Primers used in PCR and their annealing temperatures are represented in Table 1, and were designed to amplify only one of the two genomic strands. Roughly, PCR was done with 30 cycles of 94°C for 60 s, 46–57°C for 60 s and 72°C for 20 s. All PCRs were finished with one cycle of 72°C for 10 min. The amplified PCR products were cloned into pGEM-T Easy vector (Promega). A part of individual clones were sequenced using automatic sequencer (ABI Prism 377) and complete conversions of genomic cytosines to uracils were confirmed. A tenth volume of the first PCR products was reamplified under the same condition in the presence of 2 μCi [^{32}P]dCTP, purified and concentrated with the Wizard DNA purification kit (Promega). About 100 ng of labeled DNA was digested with 20 U of restriction enzymes *Taq*I, *Acc*II, or *Aci*I (NEB) at the proper temperatures for 12 h, resolved on 5% or 8% polyacrylamide gels, dried, and exposed to autoradiographic film.

3. Results and discussion

3.1. Unstable inheritance of DNA methylation in euchromatic repeated DNA and single-copy gene sequences during ageing

We examined the methylation status of various genomic regions in two types of fetal bovine fibroblasts, both of which were derived from a single fetus. One cell population (BEF) was relatively fresh fibroblasts at 6 PDL, while the other population (T-BEF, more than 33 PDL) consisted of BEF-derived fibroblast cells pooled from 10 different neomycin-resistant

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Abbreviations: LINE, long interspersed element

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(Neo^R) clones after transfection. During increase in the clonal population, typical phenotypes of senescence were observed in these cloned cells, such as declined growth rates (1 PDL/week), larger and more irregular cell shapes, and trypsin resistance (data not shown) [8]. Genomic DNAs isolated from both groups of fibroblasts were treated with bisulfite [7]. To investigate the methylation of single-copy genes, we amplified the promoter regions of tissue-specific genes, such as the epidermal cytokeratin gene, the mammary-gland-specific β-lactoglobulin gene, and the interleukin-13 (IL-13) gene, and resultant PCR products were digested with either *Aci*I (5′-GCGG-3′; cytokeratin gene) or *Taq*I (5′-TCGA-3′; β-lactoglobulin gene and the IL-13 gene) (Fig. 1B–D). All the promoter regions of these genes appeared to be considerably methylated in the BEF population, reflecting their functional inactivity in fibroblasts. In T-BEF, the hypermethylation status was shown to be modified; by a loss of methylation during senescence, all or a part of the enzyme recognition sites became resistant to enzyme reaction, producing various sizes of incompletely digested DNA fragments in the T-BEF cells.

Next, euchromatic repeats, such as 18S ribosomal RNA (*rRNA*) and *art2* sequences, a subfamily of bovine short interspersed sequences, were examined (Fig. 1E,F). Both sequences in the BEF group revealed moderate (18S rRNA) to high levels (*art2*) of methylation. In aged cells, methylation was changed such that a larger proportion of PCR products of both sequences was shown to be resistant to enzyme digestions. Thus in ageing bovine fibroblasts, all the examined euchromatic sequences constantly showed a decrease in methylation level, which agrees well with previous reports describing a reduction in DNA methylation level with passages of cultured human and mouse cells [9–11]. This suggests that a loss of 5-methylcytosine with passages of culture is a general phenomenon shown in cultured mammalian cells.

3.2. Stable maintenance of DNA methylation in bovine heterochromatic satellite DNA regions during senescence

Based on these observations, we further analyzed heterochromatic DNA sequences to examine whether similar epigenetic changes occur in these highly inactive chromosomal regions (Fig. 2). Three kinds of satellite sequences were examined: satellite I, II and alphoid satellite. From the restriction analyses, no apparent changes in methylation level were detected between BEF and T-BEF (Fig. 2B–D). In addition, the *Bov-B* sequence, a subfamily of bovine long interspersed elements (LINEs), was analyzed (Fig. 2E). LINE repetitive sequences are known to localize preferentially in transcriptionally inactive, dark G bands of metaphase chromosomes in human [12] and mouse [13]. It is also known that LINE sequences participate in forming heterochromatin-like structures in mammalian species [14–16]. The *Bov-B* sequence also stably maintains its epigenetic status during ageing. These results indicate that methylation patterns of various heterochromatic DNA regions are stably inherited without apparent epigenetic changes during senescence, which is in great contrast to those euchromatic sequences that showed unfaithful inheritance of DNA methylation during ageing. Therefore, these results may provide evidence for the occurrence of differential epigenetic modification among different genomic loci during ageing.

3.3. Analysis of individual Neo^R clones confirms the occurrence of differential epigenetic modification between euchromatic and heterochromatic DNA sequences

To confirm the occurrence of differential epigenetic changes in genomic DNA of ageing cells, Neo^R clones were individually analyzed (C1–C5). Heterogeneity in methylation pattern was unambiguously seen among different Neo^R clones in both promoter regions of the epidermal cytokeratin gene and the

Table 1
Primers used in PCR for amplification of various target sequences

DNA region	Primer (5′-3′)	Annealing temperature (°C)	Reference
Cytokeratin gene	CCTCTTTCTACCAAACAAACCAA (1st) ACAAACCAAAAACTAATAATACCTCA (2nd) GTGGAYGGTAAGTTATTTAAAAGGAG	55	[22]
β-Lactoglobulin gene	ACCATTTTTACTACCCTAACTAAACA TGTGATTGTGGTTTTGGGTAAAGG (1st) AGTTTGGGGYGAGGGTGATATG (2nd)	55	U31361.1[a]
Interleukin-13 gene	CTATCTCTCCAACATTTCTAATA (1st) AATACTAAAATTCCAACTAACATAAT (2nd) TTGTGGATTTATTATTGAGAAGG	55	[23]
18S rRNA	AACTCTTTCRAAACCCTATAAT (1st) ATAAATCCACTTTAAATCCTTC-3 (2nd) GGGATATTTAGTTAAGAGTAT	46 (1st) 48 (2nd)	AF176811[a]
Art2	TTAAATTCAATTCAATCACTCAATCA (1st) CCAAACCTCCCTATCCATCTC (2nd) TTTATGTGAAGAGTTGATTTATTGGA	55 (1st) 57 (2nd)	[24]
Satellite I	AATACCTCTAATTTCAAACT TTTGTGAATGTAGTTAATA	46	[25]
Satellite II	CAACCCATAATCAATAAACTC (1st) AACAATTACTTTAATCCCAAATTA (2nd) GTTGAGGTAGTAGTTAGGTA	48 (1st) 50 (2nd)	[26]
α-Satellite	AATAATTCCACATTCCRTAAAACCC (1st) CCRATCCCCTCTTAATAAAAACC (2nd) GATGTTTTYGGGGAGAGAGG	55	AJ293510[a]
Bov-B	TTCAATTCAATCTCAATCATATC ATGTTGGGAAAGATTGAGGGTA (2nd) ATTGAGGGTAGGAGGAGAAG (2nd)	55	[27]

1st: primers used in primary PCR; 2nd: primers in hemi-nested PCR.
[a]GenBank accession number.

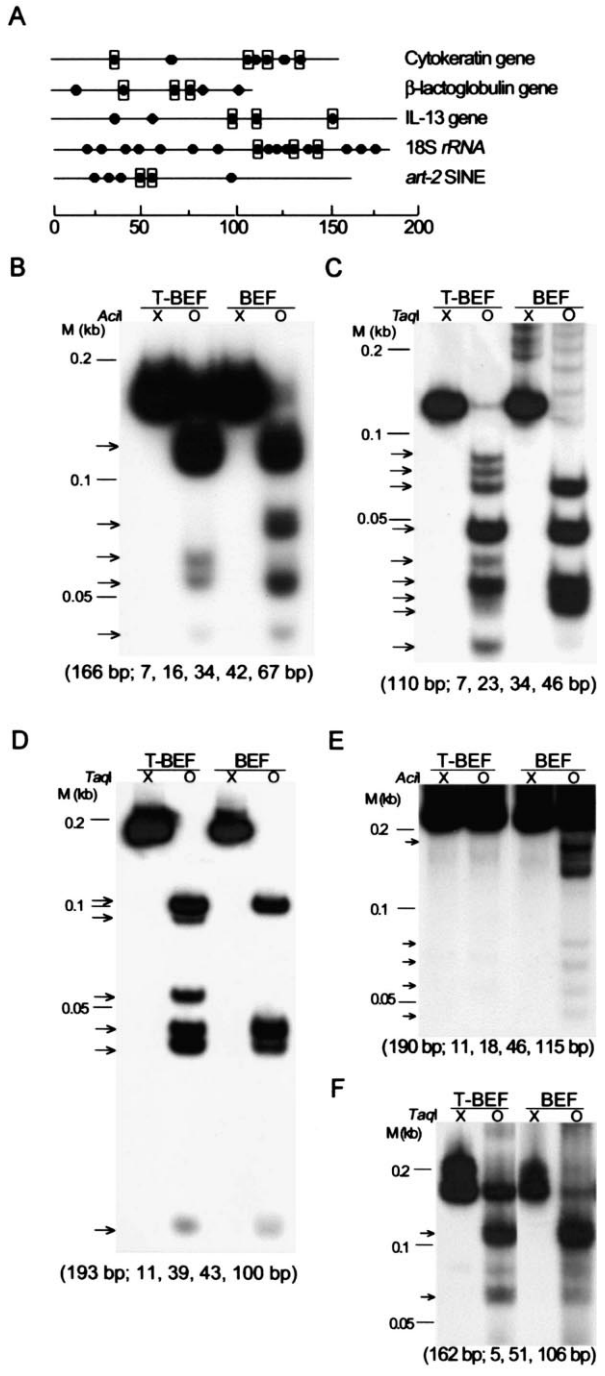

Fig. 1. Unstable inheritance patterns of DNA methylation in single-copy gene sequences during senescence. A: Schematic representation of amplified DNA fragments showing relative locations of CpG dinucleotides (closed circles) and restriction enzyme recognition sites (rectangular boxes). The size of DNA fragments produced from complete digestion of PCR products by restriction enzyme is represented below the restriction panels. B–F: Restriction enzyme analyses of PCR products amplified from promoter regions of the bovine epidermal cytokeratin gene (B), the mammary-gland-specific β-lactoglobulin gene (C), the IL-13 gene (D), the 18S *rRNA* region (E; *Aci*I enzyme), and the *art2* sequence (F; *Taq*I). DNA size markers (M) are denoted in kb along with the positions of digested DNA fragments (arrows). X, intact PCR products; O, enzyme-digested PCR products.

β-lactoglobulin gene (Fig. 3A,B), as described previously [17]. Individual clones revealed, as a whole, relatively lower methylation compared with the BEF cells and the observed variations appeared random. However, the methylation status of the α-satellite region was quite similar among different individual Neo^R clones and to the parent BEF population (Fig. 3C), indicating a stable inheritance of the α-satellite sequences during ageing. Such a stable methylation pattern was also seen from analysis of the satellite II region (data not shown). The *Bov-B* LINE sequences also showed a stable methylation pattern among the different Neo^R clones (Fig. 3D). This study with individual Neo^R clones further confirmed the facts that differential epigenetic modifications occur between euchromatic and heterochromatic DNA sequences, and that the methylation status of heterochromatic sequences is more resistant to epigenetic modification in aged cells. These findings

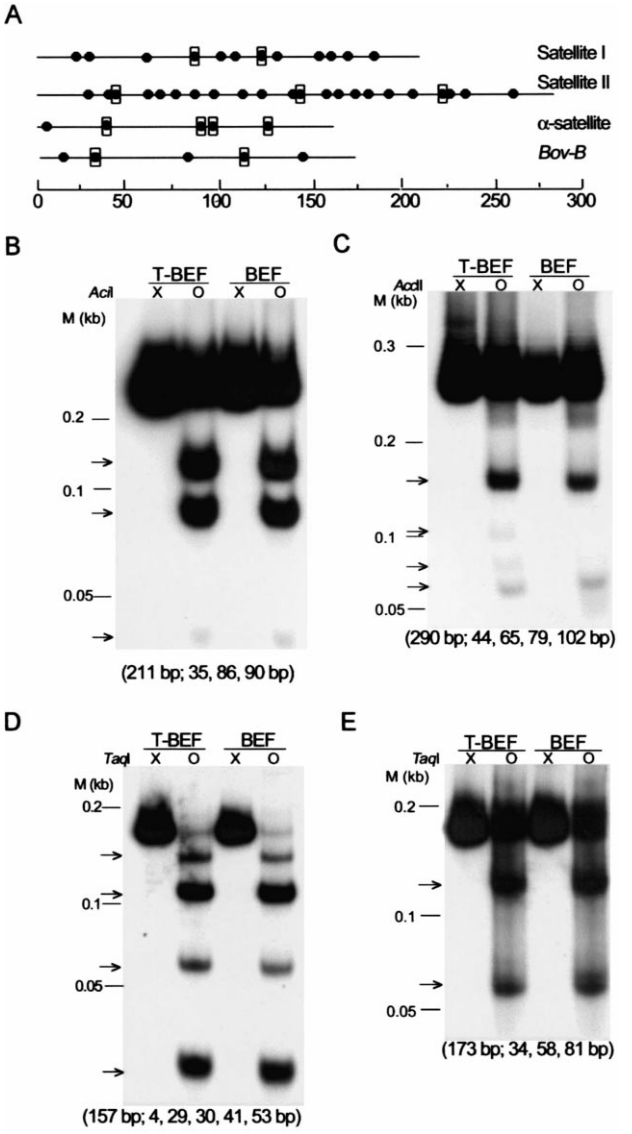

Fig. 2. Stable maintenance of DNA methylation level in various heterochromatic DNA sequences during senescence. A: Schematic diagram of amplified DNA fragments. *Aci*I, *Acc*II and *Taq*I restriction analysis of PCR products amplified from bisulfite-treated genomic satellite I region (B, *Aci*I), satellite II region (C, *Acc*II), α-satellite DNA region (D, *Taq*I) and the *Bov-B* sequence (E, *Taq*I), respectively.

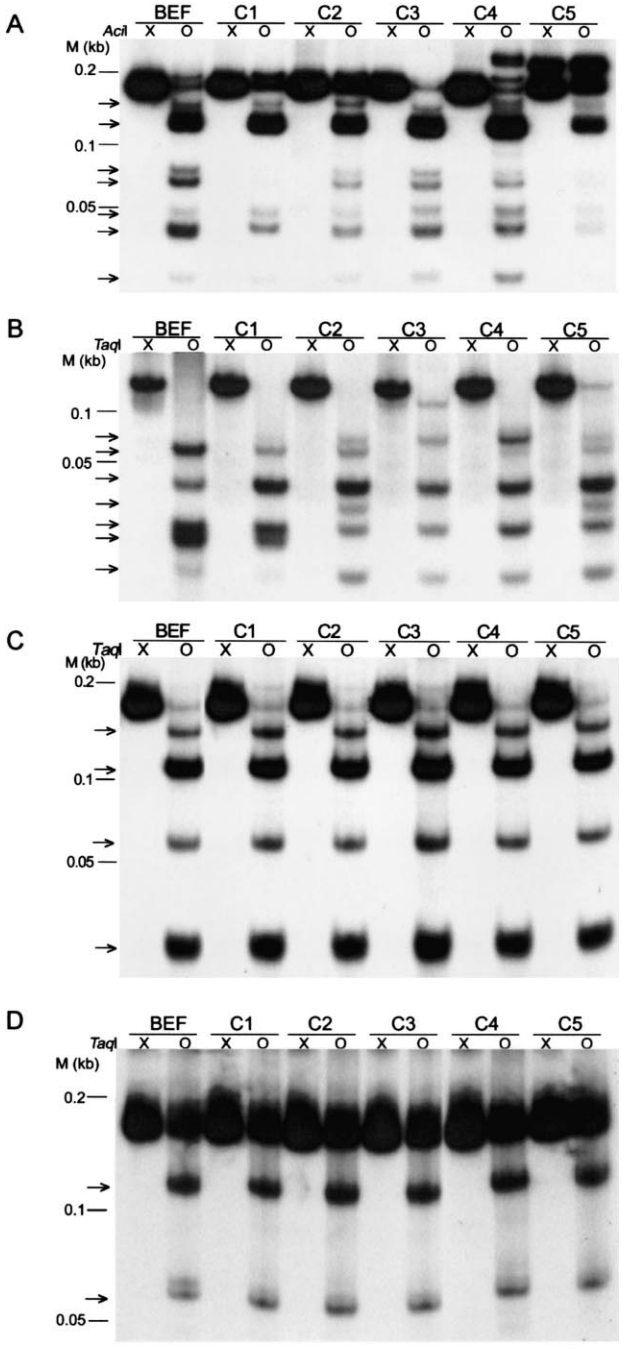

Fig. 3. Inheritance patterns of DNA methylation in individual Neo^R ageing T-BEF clones. PCR products amplified from bisulfite-treated genomic DNAs of individual Neo^R T-BEF clones (C1–C5) were digested with *Aci*I (A, the epidermal cytokeratin gene promoter region) and *Taq*I (B, the β-lactoglobulin gene; C, the α-satellite DNA sequences; D, the *Bov-B* LINE sequences).

suggest that the reduction in DNA methylation that is associated with ageing is restricted to euchromatic DNA sequences and thus it may not be a global phenomenon occurring throughout the whole genome.

Our results indicate that euchromatic DNA sequences unfaithfully inherited their DNA methylation status during the ageing process, whereas heterochromatic DNA sequences such as satellite DNA regions stably maintained their own methylation. A reduction in methylation level during the ageing process is not a new observation. The novelty of our results lies in the different inheritance modes of DNA methylation between euchromatic and heterochromatic DNA regions. Our results came from the examination of nine genomic loci using a combined bisulfite–PCR technique [18]. Usually, senescence-related methylation changes have been studied on only a limited number of genomic loci using varied sources of genomic DNAs, and the resultant heterogeneous observations do not allow a clarification of the age-dependent epigenetic modification pattern. Our global, multi-locus analyses with a single culture system make it possible to find the tendency of differential epigenetic modification between genomic DNA regions during ageing. We believe that our observation would be a clue to put the varied findings on age-related methylation changes in order.

Our findings of differential epigenetic modifications between euchromatic and heterochromatic DNA sequences are based on the experiments with in vitro cultured cells, and thus it is necessary to test whether a similar trend is observed in aged animals. However, there are reasons why our observations cannot be treated simply as vagaries seen in cultured cells. First, we examined multiple genomic loci, six for euchromatic and three for heterochromatic DNA regions, and all these regions followed, in a highly consistent manner, either the unfaithful or the stable epigenetic inheritance mode according to their euchromatic or heterochromatic locations, respectively. Second, a loss of methylation in single-copy genes is a commonly observed phenomenon related to ageing [19–21]. Our results also showed the same trend of reduction in methylation level at the tissue-specific gene loci (Fig. 3). Third, through individual analysis of the 10 independent Neo^R clones for methylation status, we confirmed again the rule of differential epigenetic modification between euchromatic and heterochromatic DNA regions (Fig. 3). The Neo^R clones were collected from two separate transfection experiments. Nevertheless, they showed very similar methylation levels in the α-satellite, satellite II and *Bov-B* LINE sequences, which could not be considered a false result presumably observed in studies with cultured cells. Therefore, considering all these reasons, the event of differential epigenetic modifications among different genomic loci is, as we believe, a phenomenon probably not limited to in vitro cultured cells.

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