

High resolution methylation analysis of the galectin-1 gene promoter region in expressing and nonexpressing tissues

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Abstract We conducted by bisulfite genomic sequencing a high resolution study of the methylation of the galectin-1 gene in expressing and nonexpressing tissues. We show that: (i) hypomethylation of galectin-1 promoter correlates with expression; (ii) differences in methylation occur in a small region, which include a CpG cluster; (iii) the density of methyl-CpGs rather than site-specific methylation distinguishes the nonexpressing from the expressing alleles; (iv) the modification profiles in nonexpressing tissues are highly heterogeneous; (v) a single CpG within 1300 bp is always methylated both in expressing and nonexpressing tissues; (vi) these features are conserved in rat and mouse.

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Key words: Galectin; Promoter; DNA methylation; Transcription regulation

1. Introduction

Galectin-1 is one of the best characterized galectins, a family of animal lectins that share binding specificity for β -galactoside containing carbohydrates [1,2]. A variety of biological functions, both during development and in adult tissues, has been attributed to galectin-1. Its role in cell-cell and cell-matrix interactions is well established [1]. However, a body of evidence suggests that galectin-1 is also involved in the regulation of cell growth [3,4], in neoplastic transformation [4–8], in myoblast differentiation [9] and in the maturation of T-lymphoblastoid cells [10]. Recently, galectin-1 has been identified as a mediator of T-cell and thymocyte apoptosis [11], as an essential factor for neurite outgrowth and correct axonal guidance in the developing olfactory system [12] and as a required factor in the splicing of nuclear pre-mRNA [13].

The galectin-1 gene is developmentally and tissue specifically regulated [1,14]. In liver, thyroid, cerebellum and brain, galectin-1 mRNA levels are barely detectable while galectin-1 expression is very high in heart and in skeletal muscle [1,15]. Abnormal galectin-1 expression has been found during cell dedifferentiation and in neoplastic cells. In general, galectin-1 expression increases several fold with loss of differentiation [8,16,17] or with cell transformation [1,5,7,8,18] thereby conferring a high metastatic potential on neoplastic cells [5,19].

The molecular mechanisms governing modulation of the

transcriptional activity of the galectin-1 gene have only recently been investigated [20–22]. A small region (from position –50 to +50) of the galectin-1 promoter is sufficient to drive efficient transcription. Moreover, galectin-1 promoter constructs, containing up to 2500 bp upstream from the transcription initiation site, are transactivated by factors present in expressing and nonexpressing cells [21]. We previously found a positive correlation between hypomethylation of the galectin-1 promoter region and galectin-1 expression in various cell lines. We also showed that reactivation of the silent galectin-1 alleles in cell hybrids [20] is accompanied by a transition from a fully methylated to a fully unmethylated state of several CpG dinucleotides in the promoter region [22]. A controversial issue of this kind of study is whether changes in the pattern of DNA methylation observed in cultured cells reflect physiologically relevant processes occurring in animal tissues [23,24]. To address this question we defined the precise methylation map of the gene in various rat and mouse adult tissues. We found that the methylation state correlates with gene expression also in tissues. However, while in cell lines differences in methylation were spread over a large area, in tissues differences in CpG modification were confined to a cluster of 11 CpGs surrounding the transcription start site. In addition, while in cell lines methylation was an all or none phenomenon [22], nonexpressing tissues exhibited highly heterogeneous methylation profiles and a single CpG site was constantly methylated also in expressing tissues. The detailed analysis of the methylation state of quite a large region of the galectin-1 gene represents a useful contribution to studies of the relationship between methylation and transcription and may also provide insight into the mechanisms by which DNA methylation regulates gene expression in tissues.

2. Materials and methods

2.1. Isolation of nucleic acids from tissues

The rodents used in this study were Sprague-Dawley rats and C57/B16 mice. Genomic DNA was prepared from freshly minced tissues by a standard phenol-chloroform extraction method [25].

2.2. CD-PCR

Cytosine deamination by bisulfite treatment of single-stranded DNA and subsequent PCR amplification (CD-PCR) was done essentially as described by Frommer et al. [26]. An 8- μ g sample of genomic DNA was first digested with *Eco*RI or with *Xba*I and then denatured in 0.3 M NaOH for 15 min at 37°C in a volume of 100 μ l, and then 60 μ l of 10 mM hydroquinone and 1.04 ml of 3.6 M sodium bisulfite (pH 5) were added. The reaction mixtures were incubated at 50°C for 16 h in the dark. The DNA was desalted and concentrated with GeneClean (Bio101), desulfonated with 0.3 M NaOH for 15 min at 37°C, neutralized with 3 M ammonium acetate (pH 7) and ethanol precipitated. Unmethylated C is changed to U while m⁵C remains as

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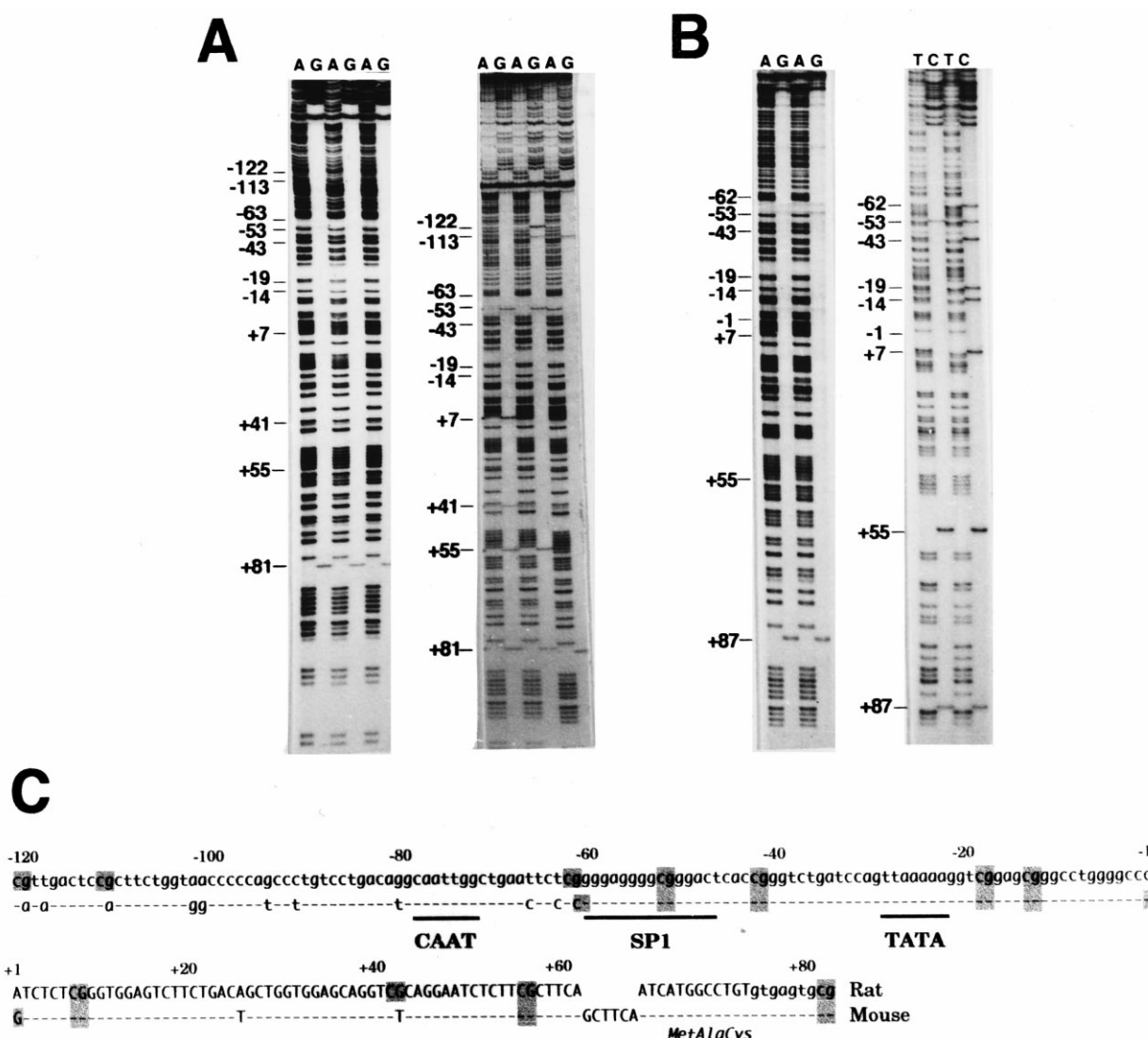


Fig. 1. Analysis of clones derived from the galectin-1 promoter region after bisulfite treatment. A: Methylation analysis of representative clones derived from the top strand of the galectin-1 promoter region of rat skeletal muscle (left) and brain (right). B: Methylation analysis of representative clones derived from the top strand of the galectin-1 promoter region of mouse skeletal muscle (left) and from the bottom strand of mouse brain (right). Genomic DNAs from the different tissues were treated with sodium bisulfite, amplified, and cloned into the pCRII vector. Plasmids were sequenced reading the upper strand of the original DNA in the 3'-to-5' direction and the lower strand in the 5'-to-3' direction. U's, derived from unmethylated C's appear as T's (lower strand) or as A's (upper strand) while m⁵C's appear as C's (lower strand) or as G's (upper strand). C: Alignment of the rat and mouse galectin-1 promoter sequences. Identical residues in the mouse sequence are indicated by dashes, the CpG dinucleotides are shaded, and the consensus elements are underlined. Numbering refers to the rat sequence, the transcribed sequence corresponding to the first exon is in capital letters and the amino acids corresponding to the first three coding triplets are indicated.

2.3. Determination of primers for CD-PCR

method [28] with a T7 sequencing kit (Pharmacia). Primers U2, U1, L1 and L2, used in a previous study [22], correspond to the following regions: positions -205 to -177 and $+93$ to $+120$ for the amplification of the upper strand and positions -124 to -97 and $+87$ to $+114$ for the lower strand. New oligonucleotides (U3, U4, U5 and U6) were designed as complementary to sodium bisulfite-treated rat DNA according to the criteria specified by Frommer et al. [26]. Modified primers were: U3 from positions -199 to -169 (5'-ACCCCTCTC-TTAAATCCCTCCCTCAAATCC-3'); U4 from positions -618 to -588 (5'-GGTGTAGTTAGTTTGTAGTGAGTAGTATTG-3'); U5 from positions $+725$ to $+754$ (5'-CATTACAC ACAACTACCTAAATAAATACAC-3') and U6 from positions $+295$ to $+323$ (5'-TGAGTTGTGGTATGTTTGTGAAGGTTAG-3'). Primers, chosen on the basis of the mouse genomic sequence [15], correspond to the following regions: positions $+98$ to $+125$ and -205 to -178 for the amplification of the upper strand and positions -124 to -97 and $+92$ to $+119$ for the lower strand. Modified primers were: MU2 (5'-GTGTTAGGATTTTGAGGGAGGGGTTAGG-3'); MU1 (5'-TT-

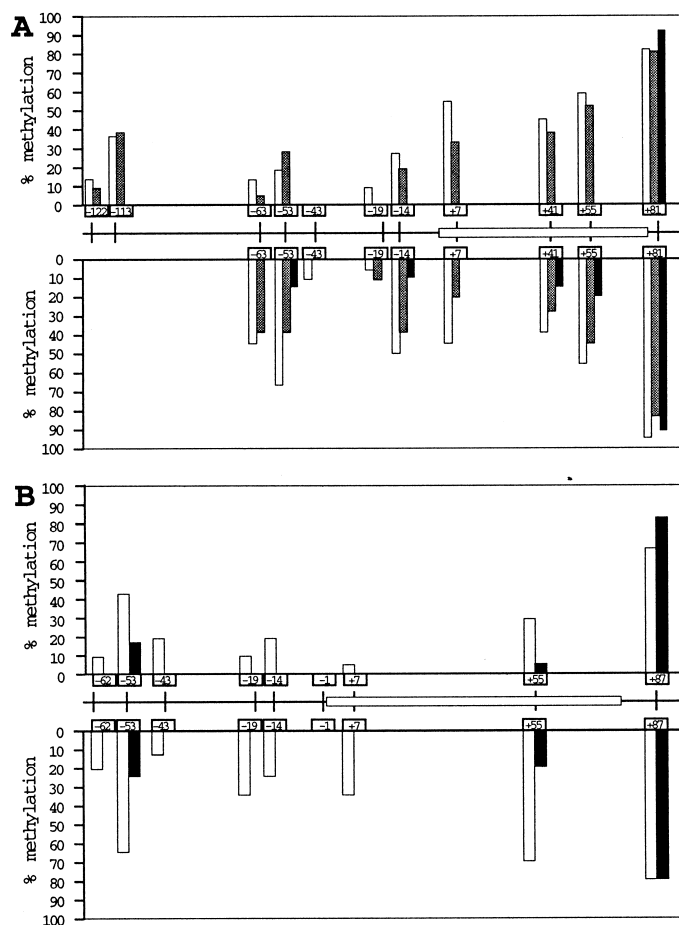


Fig. 2. Percentage of DNA methylation at individual CpG sites in the galectin-1 promoter. Data obtained from genomic sequencing of bisulfite-treated DNA were compiled by individual CpG dinucleotides and expressed as the ratio of methyl-C to the number of clones. A: Rat tissues: brain (open bars); liver (gray bars); and skeletal muscle (black bars). The relative position of CpG dinucleotides (vertical lines) and of the first exon (open box) of the rat galectin-1 gene is indicated between the upper (top strand) and the lower (bottom strand) panels. B: Mouse tissues: brain (open bars); skeletal muscle (black bars). The mouse galectin-1 region is depicted as in A.

CTACTCAATCCCCTAAACCTTAAAAC-3'); ML1 (5'-TCCATA-AACTCCACTTCTAATAACCCCC-3') and ML2 (5'-TTAGTTTTT-TAGATTTTGGGAATAGAGGG-3').

2.4. *In vitro* DNA methylation and gel-retardation assay

The galectin-1 promoter fragment from positions -50 to +50 was obtained by excision from the pGAT50 plasmid [21]. The fragment (10 µg) was treated with 8 U of *Sss*I methylase (New England Biolabs) at 37°C in the presence of 5 mM adenosylmethionine for 8 h. Complete methylation of treated plasmid was confirmed by *Hpa*II restriction enzyme digestion. Methylated and unmethylated fragments were used as probes for binding reactions. The labelling reaction was performed with T7 polynucleotide kinase (Boehringer-Mannheim) and [γ -³²P]dATP. Gel shift assays were performed as described elsewhere [29]. Samples were loaded on 5% nondenaturing polyacrylamide gels. The gels were then dried and subjected to autoradiography.

3. Results

3.1. Methylation of CpG sites in the galectin-1 promoter region

We examined the methylation state of the galectin-1 gene promoter region in rat tissues in which galectin-1 expression was very high (skeletal muscle) and very low (brain and liver) ([1,15] and data not shown). The promoter regions of the rat, mouse and human galectin-1 genes contain a conserved cluster of CpG dinucleotides surrounding the transcription initiation site [22]. Fig. 1C shows an alignment of the rat and mouse

sequences complete with their relevant features including the relative position of CpG sites. The methylation state of all the CpG doublets of the cluster of the rat gene from positions -122 to +81 was analyzed in the different tissues by genomic sequencing. This region was previously found to be fully methylated at every CpG site in nonexpressing rat liver cell lines and fully unmethylated in human osteosarcoma expressing cells and in reactivated rat alleles in cell hybrids [22]. We used two sets of primers each specific for the upper (U1 and U2) and the lower (L1 and L2) strands of bisulfite-treated DNA for the analysis of the methylation state of 11 and 9 dinucleotides, respectively. DNAs from the nonexpressing liver and brain, and from expressing skeletal muscle were subjected to CD-PCRs. The amplified fragments were cloned and several plasmid clones derived from each tissue were analyzed by DNA sequencing. Representative sequence profiles of rat skeletal muscle and brain are presented in Fig. 1A; the statistical analysis of all the experiments is shown in Fig. 2A. The galectin-1 promoter region was consistently methylated in brain and liver, and mostly unmethylated in skeletal muscle. However, the methylation profiles of the CpG sites in rat tissues were more complex than those observed in cell lines [22]. In particular, sites at positions -122, -113, -63, -53, -14, +7, +41, +55 on the top strand were fully unmethylated

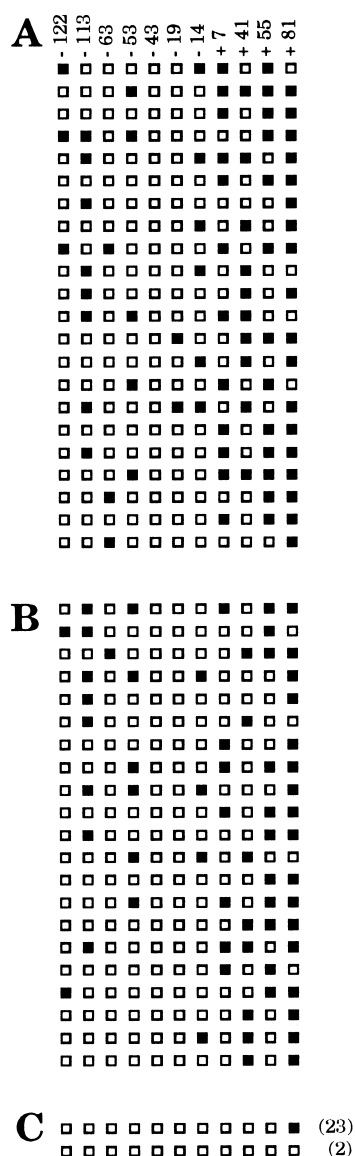


Fig. 3. Variation in DNA methylation in individual PCR clones from the galectin-1 promoter. All the sequenced molecules from rat brain (A), liver (B) and muscle (C) upper strands are shown. Number of clones from muscle exhibiting the same pattern is indicated in parentheses. Numbers on top show location of CpG dinucleotides (boxes). Black boxes indicate that the corresponding cytosines are methylated.

in muscle and variably methylated in brain and liver. A very low level of methylation compared with that observed in brain and liver was present at some sites on the bottom strand in skeletal muscle. The sites at positions -43 and -19 were unmethylated or hypomethylated on both strands in expressing and nonexpressing tissues. Strikingly, the last site of the cluster, at position $+81$, was almost fully methylated in all tissues. The methylation pattern of the top and bottom strands was almost symmetrical in each of the different tissues (Fig. 2A).

With the bisulfite genomic sequencing technique, each plasmid clone is representative of a single allele each derived from a different cell [26]. Therefore, by this procedure it was possible to appreciate a high heterogeneity of the methylation patterns within the nonexpressing tissues (see examples shown

in Fig. 1A Fig. 3A and B). Fig. 3 shows the methylation patterns of each individual molecule analyzed for rat brain (A), liver (B) and muscle (C) top strands. It is apparent that there was no correlation between the methylation at one site and methylation at other CpGs in the same DNA strand and that methylation of a site is independent of its neighbors. Moreover, in rat brain and liver every molecule had at least two methylated CpGs whereas in rat muscle no molecule had more than one. The same features were observed for the lower strands (data not shown).

To verify whether the methylation profiles observed in rat were conserved in another species, we performed the same analysis with mouse skeletal muscle and brain using appropriate primers on bisulfite-treated mouse genomic DNAs. Representative sequence profiles of skeletal muscle and brain are shown in Fig. 1B, and a statistical analysis of all the experiments performed is presented in Fig. 2B. Although the overall level of methylation was lower than in rat, the general pattern of methylation was similar, including the high degree of heterogeneity and the random distribution of methylated CpGs (Fig. 1B and data not shown). The last site of the mouse CpG cluster at position $+87$ was again almost fully methylated on both strands in expressing and nonexpressing tissues (Fig. 2B).

3.2. Limits of the differentially methylated genomic region

To establish the spatial limits of the genomic region affected by changes in the CpG modification state we designed two sets of primers (U3–U4 and U5–U6), each specific for upper strand amplification of the galectin-1 gene region from positions -587 to -200 including 11 CpGs, and from positions $+324$ to $+724$ including 7 dinucleotides, respectively (Fig. 4). DNAs from brain and skeletal muscle were subjected to CD-PCRs and the amplified fragments were cloned. At least 15 plasmid clones for each fragment were subjected to sequencing reactions. An analysis of the entire region from positions -587 to $+724$ of rat brain and skeletal muscle is shown in Fig. 4A and B, respectively. The methylation pattern of the two tissues was similar in the genomic regions flanking the differentially methylated CpG cluster. Several sites were constantly unmethylated in both tissues, while others were infrequently ($<25\%$) and, in general, not differentially methylated. These results demonstrate that the changes in CpG methylation in expressing and nonexpressing rat tissues are confined to the CpG cluster lying around the transcription start site.

3.3. Methylation of the core promoter does not directly inhibit the binding of nuclear factors

A galectin-1 promoter fragment from positions -50 to $+50$, containing 4 CpG sites, was previously demonstrated to be

Table 1

Average of methylated sites per chromosome in the galectin-1 promoter region in rat (from position -122 to $+81$) and mouse (from position -62 to $+87$) tissues

Tissues*	Top strand	Bottom strand
Rat brain	3.6 (22)	4.1 (18)
Mouse brain	2.1 (21)	3.5 (20)
Rat liver	3.0 (21)	3.1 (18)
Rat muscle	0.9 (25)	1.5 (20)
Mouse muscle	1.0 (18)	1.2 (20)

*The numbers of clones analyzed for each tissue are reported in parentheses.

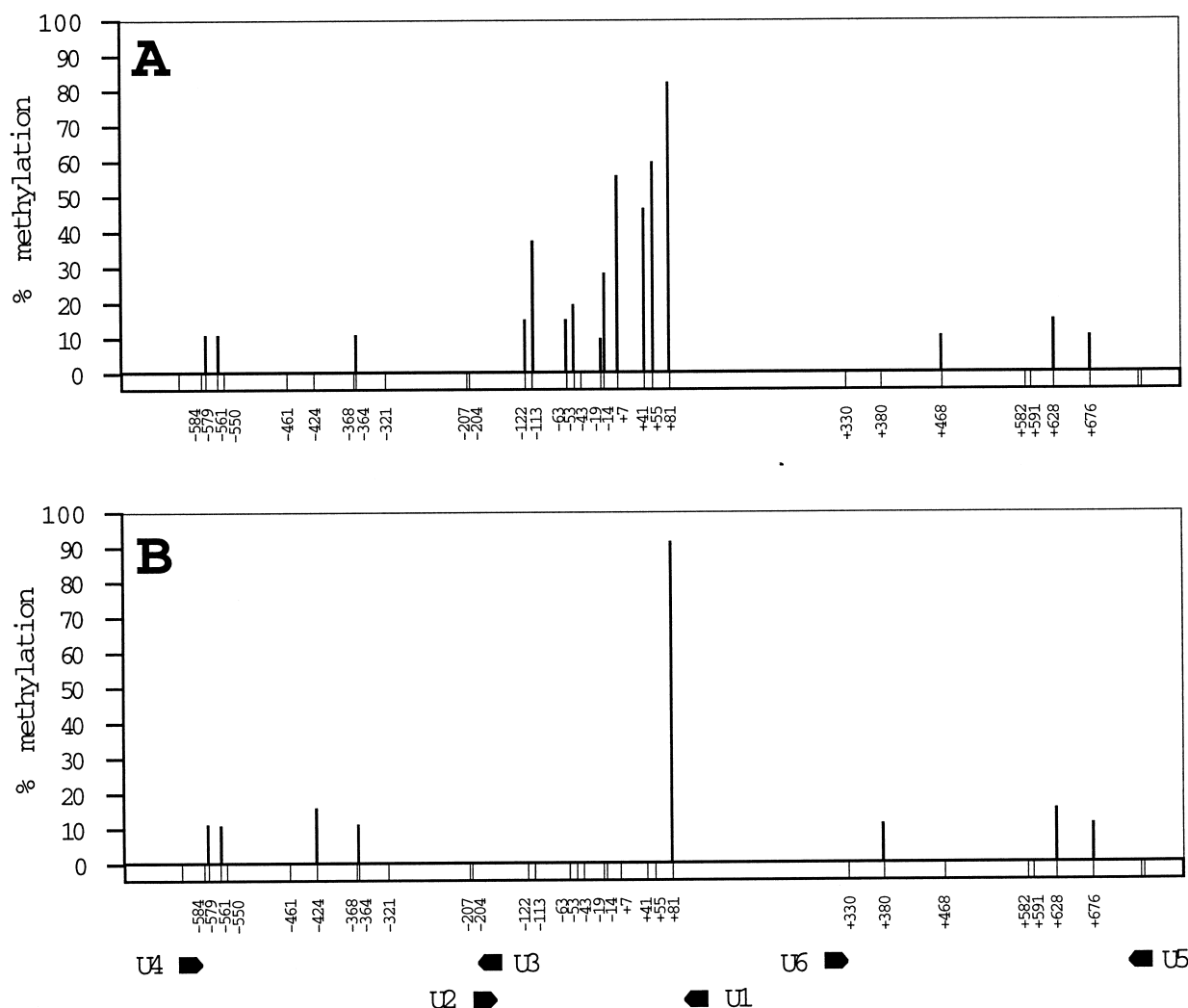


Fig. 4. Percentage of DNA methylation at individual CpG sites in the rat galectin-1 gene region from positions -587 to $+724$. Methylation analyses of rat brain top strand (A) and skeletal muscle top strand (B) are presented. The analyses shown in the figure summarize the results obtained for the region from positions -122 to $+81$ (presented in Fig. 2) and data obtained for the flanking regions. Calculations were performed and expressed as reported for Fig. 2. The positions of the 29 CpG sites analyzed are indicated at the bottom of each panel. The positions of the synthetic oligonucleotides used for the CD-PCR analysis are indicated at the bottom of the figure.

sufficient to drive efficient transcription and to bind still unidentified nuclear factors present in expressing (NIH3T3) and nonexpressing cell lines [21]. In vitro methylation of the 4 CpG sites strongly inhibited the transcriptional activity in mouse fibroblasts [22]. To investigate whether modification of CpG sites contained in the minimal promoter region interferes with the binding of transcription factors, we performed mobility shift experiments using as probes the core promoter fragment either unmethylated or in vitro methylated at each CpG site. Both probes formed a complex with the NIH3T3 nuclear extracts and the pattern of retarded bands was identical (Fig. 5, lanes 1 and 2). Moreover, the complex was specifically inhibited by the addition of a 50-fold molar excess of unlabelled -50 to $+50$ fragment both methylated (Fig. 5, lane 3) and unmethylated (data not shown). These findings indicate that the fully methylated core promoter is still able to bind nuclear factors and, therefore, that mechanisms other than simple direct interference by DNA methylation with the binding of activators must be involved in the putative methylation-mediated silencing of galectin-1 gene.

4. Discussion

We previously defined the galectin-1 gene promoter region [21] and showed that regulation of transcription in cell lines is controlled by DNA methylation [22]. The aim of the present study was to investigate whether DNA methylation correlates with transcription also in expressing and nonexpressing tissues and to establish the range of the region exhibiting differences in the methylation pattern. We used bisulfite genomic sequencing to obtain a high resolution map of the methylation state of several CpG sites that span a large portion of the galectin-1 gene. We found that in the high expressing rat and mouse skeletal muscle, most of the CpG sites in the cluster spanning the transcription start site were fully unmethylated with the notable exception of the last site which was nearly 100% methylated (Fig. 2). Because most of the CpG sites of the cluster were methylated in nonexpressing tissues, we conclude that methylation is correlated with repression of galectin-1 gene. Analysis of the entire region from positions -587 to $+724$ of the rat galectin-1 gene in different tissues

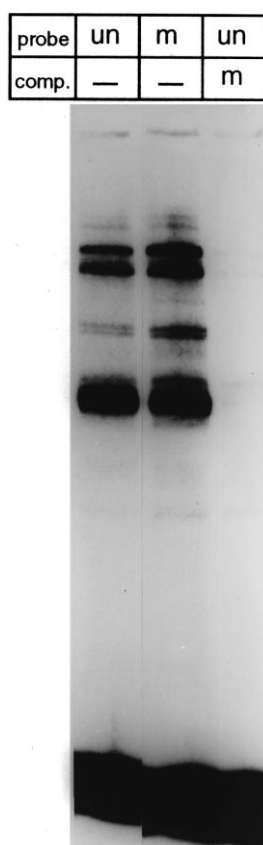


Fig. 5. Gel electrophoresis mobility shift analysis of the galectin-1 gene $-50/+50$ region. In vitro methylated (m) and unmethylated (un) fragments were end-labelled and the specific complexes formed with NIH3T3 nuclear extracts were competed by a 50-fold molar excess of unlabelled methylated fragment.

showed that differences in CpG methylation state in the expressing and nonexpressing tissues were confined to the CpG cluster encompassing the transcription start site (Fig. 4). In striking contrast, Southern blot and genomic sequencing experiments revealed that the entire region from position -587 to $+724$ was heavily methylated in nonexpressing cell lines and unmethylated in the reactivated galectin-1 alleles in cell hybrids ([22] and unpublished data).

We observed other notable differences in the methylation state of the galectin-1 promoter region between cell lines and tissues. Earlier studies on expressing cell lines and on human-rat cell hybrids [20,22] demonstrated that in the active or reactivated galectin-1 alleles, all the CpG sites of the cluster are fully unmethylated including the last site which, on the contrary, was almost totally methylated in muscle tissue. All the CpGs of the cluster are fully methylated in a nonexpressing cell line (FAO) [22]. Conversely, a variable level of methylation was observed in most of the CpG sites of the cluster in nonexpressing liver and brain tissues. The heterogeneous patterns of methylation in nonexpressing tissues might indicate that methylation contributes less in silencing of the gene in tissues compared to cell lines. Alternatively, the galectin-1 gene is regulated by methylation in both cell lines and tissues, but while in the cell lines the methylation of the gene is an all-or-none phenomenon, in tissues it is much more finely tuned.

Central to our understanding of the biological role of DNA methylation is the elucidation of mechanisms by which the

CpG modification affects gene activity, and of the mechanisms by which a cell 'decides' that specific sequences be methylated or unmethylated. It is believed that DNA methylation can influence gene activity: (i) by directly affecting the binding of transcription factors [30]; (ii) through the binding of methyl-C binding proteins [31,32]; or (iii) by altering the chromatin structure [33]. Very recently Nan et al. [34] demonstrated that a specific methyl-C binding protein, MeCP2, can act as a repressor on methylated promoters and that this activity depends on the number of methylated CpG sites within a given region. Repression first appears when the density of methylated sites in a promoter region approaches 1 per 100 bp [34]. Our genomic sequencing analysis indicates that in spite of the highly heterogeneous nature of the promoter methylation profiles the average of methylated sites in the galectin-1 promoter is over 1/100 bp in nonexpressing tissues and less than 1/100 bp in expressing tissues (Table 1). This feature is conserved in rat and in mouse galectin-1 promoter. It appears that each cell of a given tissue does not obey precise rules to control the methylation state of specific sites, but what could be critical is that, as a result of methylation and demethylation events, at least two of the 11 CpG sites in the $-122/+81$ region remain methylated in each cell and this would result in repression of the gene. This hypothesis is substantiated by the finding of a very heterogeneous methylation pattern in some of the few tissue-specific genes analyzed by genomic sequencing, i.e. prolactin and growth hormone [35]. In addition, we found that methylation does not interfere with binding of nuclear factors, even on a fully methylated galectin-1 core promoter (Fig. 5), in keeping with the proposal that methyl-C binding proteins can act as repressors without altering the binding of transcription factors [34].

Finally, an interesting finding of our study is the presence, despite the heterogeneity in the methylation profiles, of a constant full methylation of a single site within the 1300 bp region analyzed, the last of the CpG cluster located around the transcription start site (Fig. 4). This feature is conserved in expressing and nonexpressing tissues and in mouse and in rat. Thus, it is likely that the methylation of this site has a precise function. An attractive hypothesis is that a CpG site in its methylated state could be a recognition element for transacting factors, which have been postulated to be involved in regulating the methylation and demethylation of a defined DNA region [36–38].

We are currently investigating the role of methyl-CpG density in modulating the activity of the galectin-1 gene and the relative contribution of each of the different elements in defining the methylated and unmethylated state of the galectin-1 promoter region.

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