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Cell-Specific Transcriptional Regulation and Reactivation of Galectin-1 Gene Expression Are Controlled by DNA Methylation of the Promoter Region

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The galectin-1 gene is a developmentally regulated gene whose activity is strongly modulated during cell differentiation and transformation. We have previously shown that galectin-1 promoter constructs are highly active when transiently transfected in cells both expressing and not expressing the endogenous gene and that the basal activity is determined by a small region encompassing the transcription start site (from positions −50 to +50). We have now investigated the role of DNA methylation in galectin-1 gene expression. Southern blot analysis with *Hpa*II and *Msp*I endonucleases and sodium bisulfite analysis of genomic DNA from expressing and nonexpressing cell lines and cell hybrids showed a close correlation between gene activity and demethylation of the 5′ region of the galectin-1 gene. We found that the galectin-1 promoter region is fully methylated, at every CpG site on both strands, in nonexpressing differentiated rat liver (FAO) and thyroid (PC Cl3) cells and unmethylated in the expressing undifferentiated liver (BRL3A) and thyroid transformed (PC *myc*/*raf*) cell lines. In addition, reactivation of the silent FAO alleles in the FAO-human osteosarcoma (143TK$^-$) hybrid cells is accompanied by a complete demethylation of the promoter region. Finally, when galectin-1–chloramphenicol acetyltransferase (CAT) promoter constructs were methylated in vitro by *Sss*I methylase at every cytosine residue of the CpG doublets and transfected into mouse fibroblasts, the transcription of the CAT reporter gene was strongly inhibited.

Mammalian lectins are a growing class of proteins that share the presence of a carbohydrate recognition domain and are involved in several biological processes (17). Changes in the expression of endogenous lectins and abnormal glycosylation may both result in altered protein-carbohydrate interactions and are characteristic of a number of diseases, including autoimmune diseases and cancer (17). In this work, we investigated whether changes in DNA methylation, which are frequently associated with differentiation and transformation events (28, 43), can account for variations in the expression of the endogenous lectin galectin-1 (2, 3).

Galectin-1 appears to play a key role in different biological processes, such as cell growth control (37, 40, 51, 52), cell-cell and cell-matrix interactions, including acquisition of the metastatic phenotype (14, 22, 34, 50), and the maturation of T-lymphoblastoid cells (5). The expression of galectin-1 is strongly modulated during development (38) and increases dramatically with transformation and loss of differentiated functions both in cell lines (10, 41) and in mammalian tissues (9, 27). By contrast, treatment of transformed neural cells with differentiating agents leads to extinction of galectin-1 expression (7, 33). Nuclear run-on experiments showed that regulation of this gene occurs, at least in part, at the transcriptional level (10). To date, however, little is known concerning the mechanisms whereby transformation and loss of differentiation lead to transcriptional activation of the galectin-1 gene.

We and others previously demonstrated that in cell hybrids resulting from the fusion of human osteosarcoma cells (expressing the galectin-1 gene) with differentiated rat liver FAO cells (nonexpressing), the rat galectin-1 alleles are activated (8), thus suggesting that the donor cells contain regulatory factors that activate FAO galectin-1 alleles. On the other hand, galectin-1 promoter constructs are transactivated by factors present in both expressing and nonexpressing cells (45). To explain this, we postulated that the gene is repressed in nonproducing cells, through a *cis*-acting mechanism which is nonfunctional on transiently transfected promoters, and is derepressed in the hybrids in a dominant fashion. We also found that the galectin-1 gene is activated in FAO cells by treatment with the demethylating agent 5-azacytidine (8). Thus, changes in DNA methylation may be one of the mechanisms involved in the control of expression of the galectin-1 gene. To test this, by Southern blot analysis we examined the methylation status of the galectin-1 gene both in cell lines and in hybrids, and we found a positive correlation between hypomethylation of the 5′ region of the gene and its expression. In addition, we analyzed the galectin-1 promoter region by the sodium bisulfite technique (13, 20), which allows determination of the methylation state of each CpG doublet within single DNA strands and, by using appropriate primers, selective analysis of the species-specific alleles in the human-rat hybrids. Finally, we carried out transient-expression assays with galectin-1–chloramphenicol acetyltransferase (CAT) gene constructs after in vitro methylation to investigate whether methylation of the promoter region affects transcriptional activity. The results of these experiments indicate that complete methylation of a cluster of CpG dinucleotides spanning the transcription start site is sufficient for suppression and that complete demethylation of the cluster is sufficient for activation of transcription of the galectin-1 gene.

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MATERIALS AND METHODS

Cell lines and isolation of nucleic acids. Rat hepatoma differentiated FAO cells (16), rat liver BRL3A (36), rat thyroid PC Cl3, rat thyroid transformed PC *myc*/*raf* (21) cells, and mouse NIH 3T3 fibroblasts were maintained as monolayer cultures and grown in Coon's modified Ham's F-12 medium supplemented with 5% fetal calf serum. 143TK$^-$ human osteosarcoma-derived cells (ATCC CRL 8303) and hybrid clones FAO/143TK$^-$ and FAO/BRL3A (8) were grown in Eagle's modified minimal essential medium supplemented with 10% fetal calf serum and 1× nonessential amino acid mixture (Sigma). Genomic DNA was prepared from cells by a standard phenol-chloroform extraction method (46). Total RNA was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (12).

Southern and Northern (RNA) blot hybridizations. High-molecular-weight genomic DNAs (10 μg) were digested with *Msp*I or *Hpa*II restriction endonuclease. All digestions were carried out overnight with a three- to fivefold excess of enzyme. Completeness of digestion was verified by adding 1 μg of phage λ DNA to the reaction mixtures. DNA fragments were separated by electrophoresis through a 1.2% agarose gel and transferred to nylon filters. Filters were denatured in 0.4 N NaOH, neutralized in 0.2 M Tris (pH 7.5)–1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), and then hybridized in 6× SSC–5× Denhardt's solution–100 μg of sheared salmon sperm DNA per ml–0.5% sodium dodecyl sulfate (SDS). Mouse galectin-1 cDNA (11) and an *Avr*II-*Xba*I genomic fragment (from positions −350 to +450) (11) were labeled by a random priming procedure (46) and used as probes. Mouse and rat sequences have over 95% homology and cross-hybridize under high-stringency conditions. Northern blots were hybridized at 42°C in 50% formamide–5× SSC–5× Denhardt's solution–100 μg of sheared salmon sperm DNA per ml–0.2% SDS. The filters were washed in 0.2× SSC–0.1% SDS at 50°C. The integrity and relative abundance of the RNA samples were determined by ethidium bromide staining of the filters or by hybridization with a 5′-labeled oligonucleotide (5′-AACGATCAGAGTAGT GGTATTTCACC-3′) complementary to 28S rRNA.

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. (20). An 8-μg sample of genomic DNA was first digested with *Xba*I and then denatured in 0.3 M NaOH for 15 min at 37°C in a volume of 100 μl, and then 60 μl 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 (Bio 101), denatured with 0.3 M NaOH for 15 min at 37°C, neutralized with 3 M ammonium acetate (pH 7), and ethanol precipitated. An aliquot of DNA was amplified by using modified primers (see below). All PCRs were carried out in 100-μl volumes containing 10 mM Tris, 50 mM KCl, 1 mM $MgCl_2$, 5% dimethyl sulfoxide, 0.2 mM deoxynucleoside triphosphates, 10 pmol of each primer, and 2 U of *Taq* polymerase (Stratagene). The amplification cycles were as described elsewhere (13). The amplified fragments were cloned into the pCRII vector of the TA cloning system (Invitrogen), and then at least 20 independent clones for each fragment were sequenced by using T7 primer (Novagen) to determine the methylation pattern of individual molecules.

Determination of primers for CD-PCR. A rat galectin-1 genomic *Stu*I-*Xba*I fragment, corresponding to the region from positions −600 to +450, was isolated by screening a rat genomic library (Clontech). Sequencing reactions were carried out by the dideoxynucleotide chain termination method with a T7 sequencing kit (Pharmacia). Primers, chosen on the basis of the rat genomic sequence, 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. Oligonucleotides were designed as complementary to sodium bisulfite-treated DNA according to the criteria specified by Frommer et al. (20). Modified primers were U2 (5′-GTGTTTGGATTTTGAGGGAGGGATT TAAG-3′) and U1 (5′-TTCTACTCAATCCCCTAAACCTTAAAAC-3′) for the upper strand and L1 (5′-TCCATTAACTCCACTTCTAATAACCCCC-3′) and L2 (5′-TTAATTTTTTAGATTTTGGAATAGAGGG-3′) for the lower strand. Oligonucleotides used for the amplification of nontreated rat DNA were U2N (5′-GCGCCTGGACCCTGAGGGAG-3′) and L1N (5′-TCCGTTGACT CCGCTTCTG-3′) as upstream primers and ULN (5′-ACTCCCCTGTGTTCA TC-3′) as a downstream primer corresponding to regions from positions −205 to −186, −124 to −106, and +125 to +141, respectively.

Modified primers used for human DNA correspond to positions −177 to −151 and +153 to +180 for the amplification of the upper strand and positions −171 to −144 and +156 to +183 for the lower strand and were the following: hU2 (5′-GGGGTAGTAGTTTGTTATTTTGATTGG-3′), hU1 (5′-ACCACACAA CTAAAACTAAAATCTACTC-3′), hL1 (5′-ACAACTCACCACTCTAATTA ATCACCTC-3′), and hL2 (5′-TAGGTTATATAGTTGGGGTTAGAATTTG-3′).

In vitro DNA methylation, DNA transfection, and transient-expression assay. Plasmids pGAT50 and pGAT200 have been previously described (45). The control plasmid carrying the simian virus 40 promoter and enhancer upstream of the CAT gene (pSV2CAT) was obtained from Promega. Plasmids (10 μg) were 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 plasmids was confirmed by *Hpa*II restriction enzyme digestion.

NIH 3T3 cells (2.5×10^5/60-mm-diameter dish) were cotransfected with 10 μg of CAT constructs and 2 μg of the β-galactosidase expression vector pSVβ-gal (Promega) by the calcium phosphate precipitation method (24). After 48 h, cellular extracts were subjected to the CAT assay and the transfection efficiency was standardized by an assay of the β-galactosidase activity (46).

Nucleotide sequence accession number. The GenBank (EMBL/DDBS) accession number of the rat galectin-1 genomic sequence is U40624.

RESULTS

Methylation state of the galectin-1 gene in different cell lines. The galectin-1 gene is silent and heavily methylated in rat hepatoma FAO cells and is reactivated in the same cells by treatment with 5-azacytidine (8). These observations prompted us to investigate the state of methylation of the galectin-1 gene in four distinct rat cell lines. We chose to examine rat hepatoma (FAO) and rat thyroid differentiated (PC Cl3) nonexpressing cell lines and rat liver undifferentiated cells (BRL3A) and PC Cl3 cells transformed by *myc* and *raf* oncogenes (PC *myc*/*raf*), both expressing high levels of galectin-1 mRNA (8). Southern blot analysis of DNA from these cell lines using *Eco*RI, *Hin*dIII, *Pst*I, and *Msp*I restriction enzymes did not reveal any polymorphism in the organization of the galectin-1 gene (data not shown). Genomic DNAs from the four cell lines were digested with either methylation-sensitive *Hpa*II or methylation-insensitive *Msp*I and analyzed by Southern blotting. To examine the methylation status of the galectin-1 gene 5′ region, we used a mouse genomic fragment (Axm) corresponding to the region from positions −350 to +450 relative to the transcription start site (Fig. 1C). Comparison of hybridization signals obtained by probing DNA digested with *Msp*I and *Hpa*II (Fig. 1A) indicated that the galectin-1 promoter was unmethylated at CCGG sites in both BRL3A and PC *myc*/*raf* cells. By contrast, *Hpa*II digestion of FAO and PC Cl3 genomic DNAs gave rise to hybridization bands with molecular weights higher than those obtained with *Msp*I. Figure 1B shows an autoradiogram of filters hybridized with a 450-bp cDNA fragment which spans exons 2 through 4 of the galectin-1 gene (Fig. 1C). Comparison of the pattern of hybridization obtained with DNAs digested with *Hpa*II and *Msp*I indicated that although at least one CCGG site is methylated in the expressing cell lines (BRL3A and PC *myc*/*raf*), a much higher degree of methylation is present in nonexpressing cells (FAO and PC Cl3).

These results indicate that the galectin-1 gene is hypermethylated in the nonexpressing cells and reveal a positive correlation between hypomethylation and galectin-1 gene expression.

***Hpa*II-*Msp*I analysis of the galectin-1 gene in cell hybrids.** To investigate if CCGG sites within the FAO galectin-1 gene are demethylated concomitantly with its activation, we took advantage of cell hybrids resulting from the fusion of FAO cells with either human osteosarcoma expressing cells (143TK$^-$) or rat BRL3A cells (FAO/143TK$^-$ and FAO/BRL3A, respectively) (8). Almost all these hybrid clones express very high levels of galectin-1 mRNA compared with that of FAO cells (Fig. 2A) (8). The FAO/143TK$^-$ clones are interspecific hybrids, and, by using a galectin-1 probe specific for rat mRNA, we and others previously demonstrated that in all the clones analyzed the silent rat galectin-1 alleles were reactivated (8). Conversely, for three FAO/BRL3A intraspecific hybrid clones, we could demonstrate only that galectin-1 mRNA levels are higher than those in the parental BRL3A cells (8). We performed Southern blot analysis of genomic DNA from several independent hybrid clones digested with either *Msp*I or *Hpa*II, using probes that do not cross-hybridize with human 143TK$^-$ DNA (Fig. 2). The banding pattern after *Msp*I digestion of

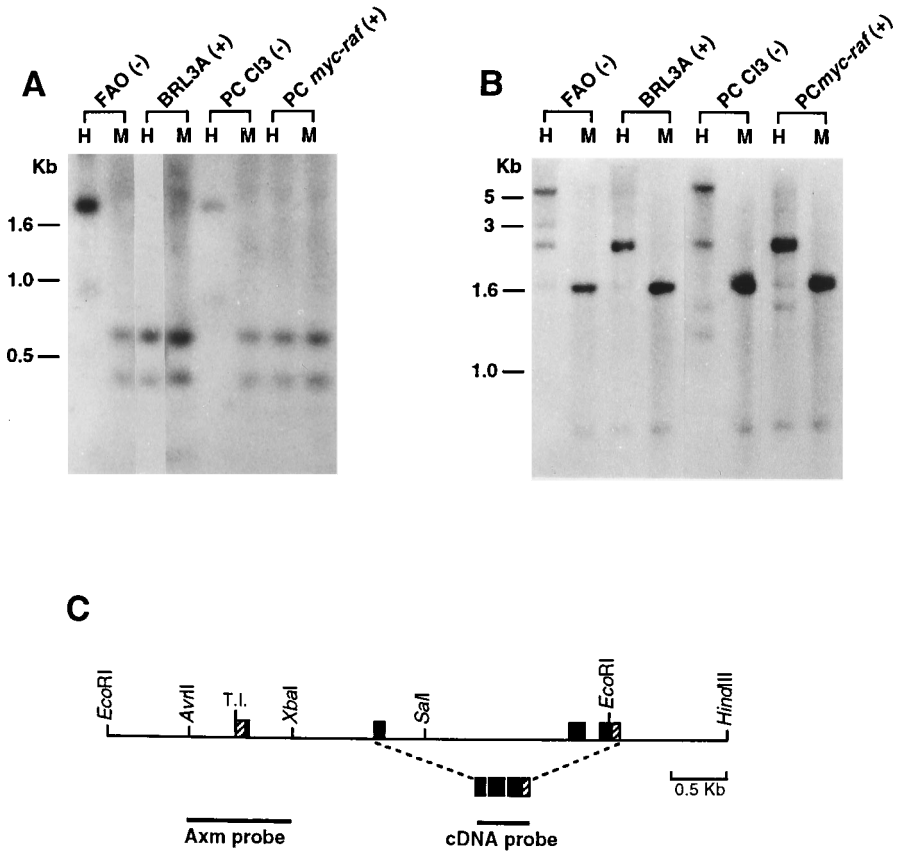

FIG. 1. Methylation pattern of *Hpa*II-*Msp*I sites of the rat galectin-1 gene in various rat cell lines. A 10-μg sample of genomic DNA from each of the indicated cell lines, either expressing (+) or not expressing (−) galectin-1, was digested with *Hpa*II (H) or *Msp*I (M) and hybridized to the Axm probe (A) and to the cDNA probe (B). A map of the genomic region including the mouse galectin-1 gene is shown (C). Coding regions (black boxes), untranslated regions (hatched boxes), and the transcription start site (T.I.) are indicated. Some restriction sites and the relative positions of the fragments used as hybridization probes are also shown. Faint bands in *Hpa*II lanes (panel B) are probably partial digestion products due to minimal residual methylation of some *Hpa*II sites within introns. The approximately 0.7-kb band is probably a genomic fragment which overlaps only partially with, and is therefore poorly recognized by, the cDNA probe.

DNA of all hybrid clones was identical to that of FAO cells (Fig. 2A and data not shown). In seven of eight hybrid clones analyzed, a nearly complete demethylation of the CCGG sites was observed with the promoter-specific Axm probe, as evidenced by the strong prevalence of the low-molecular-weight bands after *Hpa*II digestion (Fig. 2A). Interestingly, the only hybrid clone that showed consistent residual methylation of the 5′ region, FAO/BRL3A cl.3, also has low levels of galectin-1 mRNA (Fig. 2A). Figure 2B shows the autoradiograms of filters hybridized with the cDNA probe. The banding pattern for both FAO/143TK$^-$ and FAO/BRL3A hybrids was a composite of those observed with FAO and BRL3A cells or, in the case of FAO/143TK$^-$ cl.A1, more similar to that observed with FAO cells. This indicates that only partial demethylation or no demethylation occurred in the genomic region including exons 2 through 4. Thus, activation of the galectin-1 gene in cell hybrids is accompanied by complete demethylation of the *Hpa*II sites in the promoter region but not in the genomic region spanning exons 2 through 4.

Analysis of CpG sites in the gene regulatory region. To examine the state of methylation of this region in the rat cell lines and in the hybrids, first we cloned and sequenced a *Stu*I-*Xba*I genomic fragment encompassing positions −600 to +450 region of the rat galectin-1 gene (data not shown). The distribution of GpC and CpG dinucleotides in the region from positions −400 to +400 of the rat galectin-1 gene is shown in Fig. 3. Seventeen CpG sites, natural substrates of mammalian methyltransferase, are located in this 5′ region. Interestingly, the majority of these dinucleotides are clustered in a region of about 200 bp (positions −122 to +81), encompassing the transcription start site, that is sufficient for basal promoter activity (45). A similar distribution of CpG doublets is present in the corresponding region of the mouse (11) and human (23) genes (Fig. 3).

We next used a genomic sequencing method for the analysis of the methylation pattern CD-PCR (20). We designed two sets of primers each specific for the upper (U1 and U2) and the lower (L1 and L2) strands of bisulfite-treated rat DNA for the analysis of the methylation status of 11 and 9 dinucleotides, respectively (Fig. 3). To obtain information on the methylation status of 10 CpG sites lying in the corresponding region of the human galectin-1 gene in the expressing 143TK$^-$ parental cell line, two additional set of primers (hU1 and hU2 for the upper strand and hL1 and hL2 for the lower strand) specific for bisulfite-treated human DNA were designed (Fig. 3). CD-PCRs with DNAs from FAO, 143TK$^-$, BRL3A, and FAO/143TK$^-$ A1, A2, A3, and A4 clones were performed. The amplified fragments were cloned, and at least 20 plasmid clones derived from each cell line were analyzed by DNA sequencing. Typical sequence profiles of all the experiments are shown in Fig. 4. The analysis of plasmid clones derived from the upper strands showed that all 11 CpG sites were

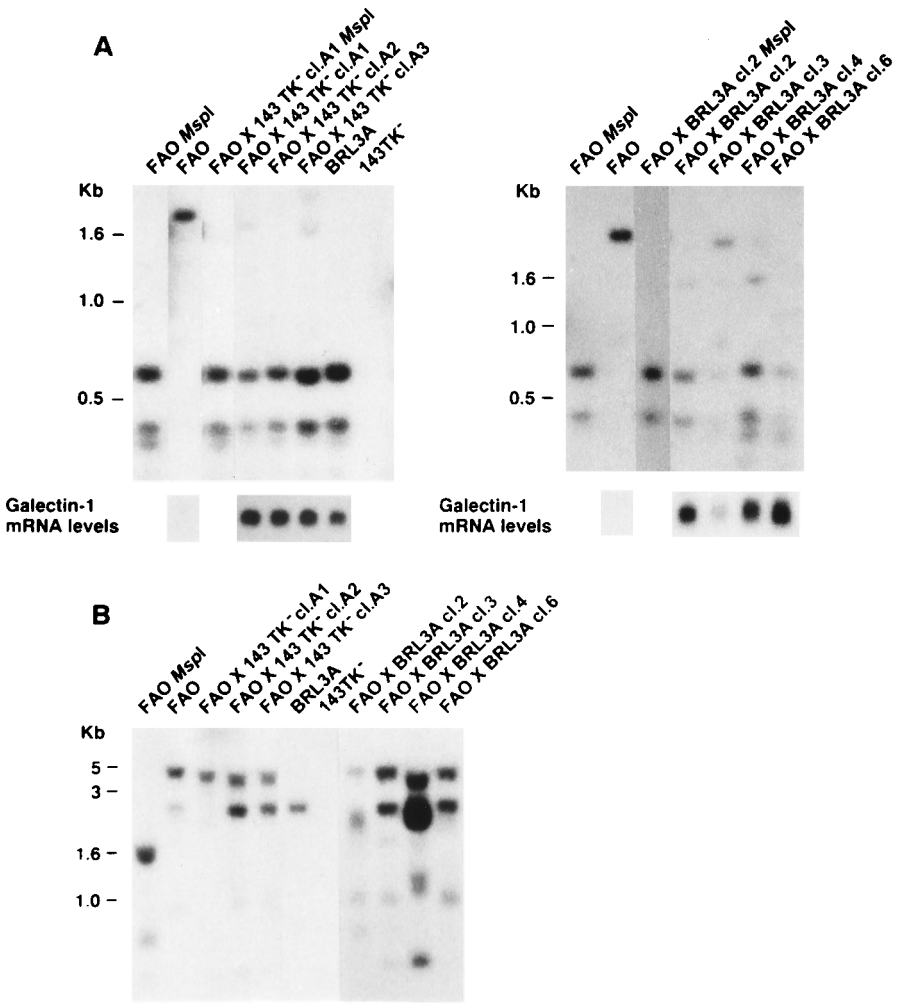

FIG. 2. Methylation pattern of *Hpa*II-*Msp*I sites of the rat galectin-1 gene in hybrids and parental cell lines. (A) Genomic DNAs (10 μg) from FAO/143TK$^-$ hybrid clones and parental cell lines (left panel) and from FAO/BRL3A clones (right panel) were digested with the methylation-insensitive *Msp*I (*Msp*I lanes) or the methylation-sensitive *Hpa*II (all other lanes) restriction enzyme and separated by 1.2% agarose gel electrophoresis. Southern blots were hybridized to the Axm probe. Galectin-1 mRNA levels were detected by Northern blot hybridization as described in Materials and Methods. (B) Southern blots of the same *Hpa*II-digested genomic DNAs were hybridized to the cDNA probe. The hybridization pattern of rat genomic DNA digested with methylation-insensitive *Msp*I endonuclease is also shown (FAO *Msp*I).

methylated in FAO cells (22 of 22 plasmid clones) and unmethylated in BRL3A cells (22 of 23 clones) (Fig. 4A and data not shown). All 10 CpG sites on the upper strand in 143TK$^-$ cells were unmethylated (21 of 21 plasmid clones) (Fig. 4C and data not shown). This is shown in Fig. 4 by the presence (FAO) or the absence (BRL3A and 143TK$^-$) of a band in lane C where a CpG (arrows) is found in the sequence of nontreated DNA. The same analysis was performed on FAO/143TK$^-$ hybrid clones. In this case, the FAO alleles of all hybrid clones were completely demethylated in all CpG sites on the upper strand (22 of 25, 23 of 23, 20 of 22, and 24 of 25 plasmid clones derived from A1, A2, A3, and A4 hybrid clones, respectively), thus reproducing the methylation pattern of expressing cells (Fig. 4A). In the few plasmids in which not all the Cs of the CpGs were converted, only one or two sites at random were nonreactive; this was likely due to a failure to deaminate by bisulfite or to a residual methylation of these sites in a small percentage of cells. The analysis of lower strands gave very similar results, and typical sequence profiles are presented in Fig. 4B and C. These results indicate that a complete demethylation of the region surrounding the transcription initiation site of the galectin-1 gene is sufficient for gene activation.

The activity of the galectin-1 promoter is inhibited by in vitro DNA methylation. Next, we sought to determine whether demethylation of the regulatory region might be an epiphenomenon that correlated with transcriptional activity or whether demethylation might play a direct role in activating transcription of the galectin-1 gene. We investigated the effects of in vitro methylation with CpG methylase on expression from two reporter plasmids transfected in NIH 3T3 cells. We used the previously described pGAT50 and pGAT200 plasmids (45), containing the promoter region from positions −50 to +50 and from −200 to +50 of the galectin-1 gene, upstream from the CAT reporter gene. These sequences drive efficient transcription when transiently transfected in NIH 3T3 cells (Fig. 5B) (45). CpG methylation of pGAT50 and pGAT200 with *Sss*I methylase (Fig. 5A) caused a 20- to 50-fold reduction of transcriptional activity (Fig. 5B). By contrast, the same treatment caused only a twofold reduction of the transcriptional efficiency of pSV2CAT control vector (Fig. 5B). In fact, it has

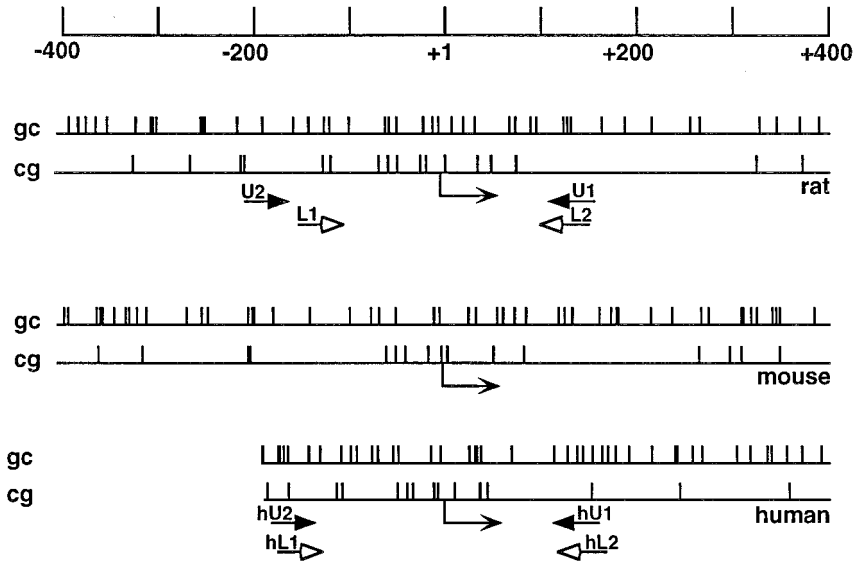

FIG. 3. Distribution of GpC and CpG dinucleotides in the galectin-1 gene 5′ region. Vertical lines indicate the position of each dinucleotide in the DNA sequence from positions −400 to +400 relative to the transcription start site (bent arrow) of the rat galectin-1 gene and in the corresponding regions of the mouse and human genes. The positions of the synthetic oligonucleotides used for the CD-PCR analysis of upper (closed arrows) and lower (open arrows) strands of rat genomic DNA (U1, U2, L1, and L2) and of human genomic DNA (hU1, hU2, hL1, and hL2) are indicated.

been demonstrated elsewhere that the simian virus 40 enhancer is only weakly sensitive to methylation (26). These results demonstrate that the transcriptional activity of the galectin-1 gene is down-regulated by DNA methylation.

DISCUSSION

Several studies have demonstrated that galectin-1 gene expression in cell lines may vary dramatically during transformation and with acquisition or loss of cell differentiation and that this protein could play a critical role in these biological processes (3). The region of the galectin-1 gene responsible for the basal promoter activity has been previously characterized (45), but no *cis*-acting elements important for modulation of galectin-1 transcription have been so far identified. In this paper, we show that DNA methylation is involved in the regulation of galectin-1 gene expression.

Cell-specific expression of the galectin-1 gene and activation of galectin-1 alleles in cell hybrids are dependent on demethylation of the 5′ region. We first found a clear correlation between transcription and the demethylation of the regulatory region in cultured cell lines. These results suggest that DNA methylation status plays a direct role in the modulation of galectin-1 transcription. That these observations are physiologically relevant and not peculiar to the experimental cell system is suggested by preliminary data which show that the galectin-1 promoter region is methylated in nonexpressing rat tissues, such as liver and thyroid tissues (44). The expression of other genes whose products are involved in developmental and differentiation programs or in the acquisition of the transformed phenotype is controlled by DNA methylation (18, 19, 28, 32, 53).

The phenomenon of gene activation in cell hybrids provides a valuable model with which to examine the changes that occur when a gene passes from an inactive to an active state. If undermethylation of specific regions in the galectin-1 gene is necessary for its expression, the reactivated alleles must become undermethylated. To test whether demethylation is a prerequisite for galectin-1 gene activation, we chose to examine somatic cell hybrids resulting from the fusion of expressing with nonexpressing parent cells in which the silent galectin-1 alleles are reactivated (8). We found that the activation of the galectin-1 alleles is always associated with the complete demethylation of the CCGG sites located at the 5′ region of the gene. Since the analysis was performed with three interspecific (FAO/143TK$^-$) and four intraspecific (FAO/BRL3A) independent hybrid clones, it is likely that this is a potentially critical event rather than an incidental event. Support for this hypothesis comes also from the observation that in FAO/BRL3A cl.3, which expresses very low levels of galectin-1 mRNA, the 5′ region of the galectin-1 gene was not demethylated.

For only a few genes has it been shown that reactivation in somatic cell hybrids is accompanied by demethylation of the 5′ region (4, 25, 47). Among these, the albumin gene is a peculiar case in that it is usually subject to extinction in hybrids but the silent alleles are both demethylated at the 5′ region and reactivated when gene dosage is in favor of expressing parent cells (48, 49). In the FAO/143TK$^-$ hybrids described here, gene dosage was balanced or in favor of nonexpressing cells (8), and both the reactivation and the demethylation of the FAO galectin-1 alleles occur independently from gene dosage.

Sodium bisulfite analysis of the promoter region. We addressed the question whether the demethylation of the promoter region of the reactivated galectin-1 gene is site specific or random or involves all sites. This was accomplished by genomic sequencing by the bisulfite technique (20). This technique relies on deamination of cytosine, but not methylcytosine, to uracil by bisulfite in single-stranded DNA. Following PCR amplification, cloning, and conventional dideoxy sequencing, methylcytosine residues are identified as cytosine and unmethylated cytosine residues are identified as thymine in the sequence ladder. The results of the sodium bisulfite analysis showed that all CpG doublets in the region from positions −122 to +81 are methylated in FAO cells and are demethylated in the FAO alleles of the four FAO/143TK$^-$ hybrid clones. It is likely that the full methylation of CpG sites

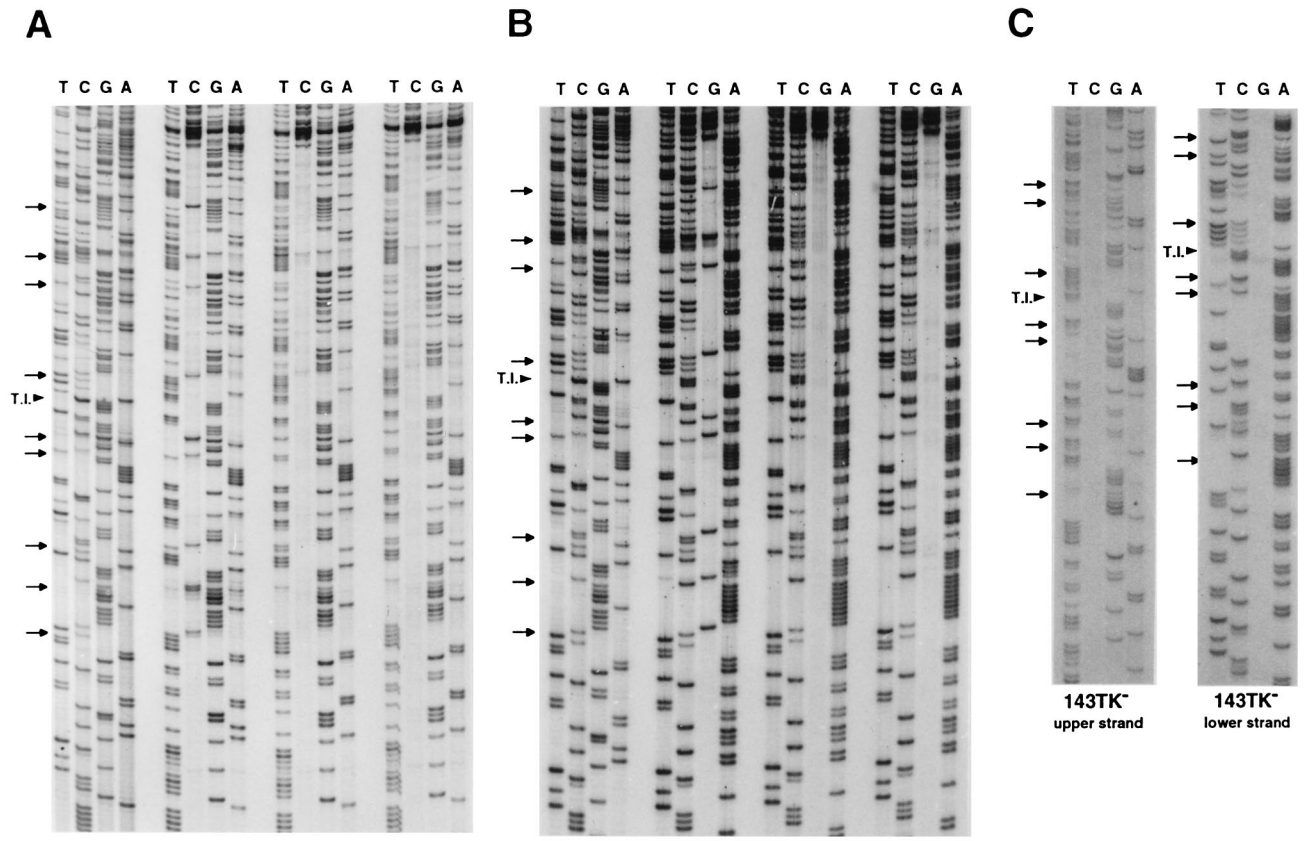

FIG. 4. Sequence analysis of plasmid clones derived from parental cell lines and hybrid clones DNA after CD-PCR. Genomic DNA was treated with sodium bisulfite, amplified, and cloned into the pCRII vector. Plasmids were sequenced with the T7 primer reading the upper strand (A) of the original DNA in the 5′-to-3′ direction and the lower strand (B) in the 3′-to-5′ direction. Thus, unmethylated C is changed to T (appearing as A on lower strands) while m^5C remains as C (appearing as G on lower strands). Sequences of representative plasmid clones are shown. Arrows indicate the Cs of nine CpG sites present in the original DNA before bisulfite reaction (lanes NT). The transcription start site (T.I.) is indicated. (C) Sequence profiles of representative plasmid clones derived from human 143TK⁻ DNA. Arrows indicate the position of the Cs of eight CpG sites present in the original DNA. The transcription start site (T.I.) is also indicated.

observed in FAO cells is mainly due to de novo methylation that frequently occurs in cell lines (1), but it is noteworthy that the reprogramming of the methylation pattern of FAO alleles in hybrids involves a complete demethylation in the regulatory region of the galectin-1 gene which acquires the same methylation pattern (fully unmethylated) observed in the BRL3A and PC *myc*/*raf* expressing cells. These results are in agreement with the observations of Murray and Grosveld (35) that site-specific demethylation in promoter regions is not sufficient to alleviate suppression and that gene suppression by methylation is region specific rather than site specific. They proposed that a minimum length of methylation-free DNA in a promoter area is the only requirement for activation of that promoter by ubiquitous transcription factors. The combined use, applied here for the first time, of somatic cell hybrids and genomic sequencing by the powerful bisulfite technique might provide an excellent system with which to study these phenomena.

The mechanisms by which demethylation is restricted to the 5′ end remain unknown, but it has been suggested that changes in the chromatin structure, appropriate for each cell type, are involved in determining the differential DNA methylation among different regions (4). If this is true, it is likely that the dominant factor that allows the reactivation of the silent galectin-1 alleles would be involved primarily in the rearrangement of the chromatin structure, which in turn would cause the loss of methylation of specific regions.

DNA methylation blocks the galectin-1 promoter. We demonstrated that in vitro methylation of the galectin-1 regulatory sequences in CAT reporter plasmids, reproducing the methylation pattern found in the FAO cells, dramatically suppressed the transcriptional potential in NIH 3T3 cells. This repression is not attributable to methylation of the CAT gene or of plasmid DNA, since the pSV2CAT plasmid, containing the methylation-insensitive SV40 enhancer and promoter (26), was much less affected by the same treatment. These data show that promoter methylation not only is associated with gene inactivity but can actually suppress galectin-1 promoter activity, thus confirming that demethylation is required for gene activation.

It has been suggested that DNA methylation can interfere with gene expression in at least three different ways (18). First, it can directly affect the binding of transcription activators or repressors (39); second, it can inhibit transcription through methyl-C-binding proteins, which bind specifically to methylated but not unmethylated DNA (6, 42); and third, DNA methylation could alter the chromatin structure, thus influencing gene accessibility (29). We did not address the question of which of these mechanisms is involved in galectin-1 gene regulation, but our data for transiently transfected methylated plasmids argue in favor of one of the first two mechanisms.

Very interestingly, it has recently been reported that expression of the human leukosialin (CD43) gene is modulated by

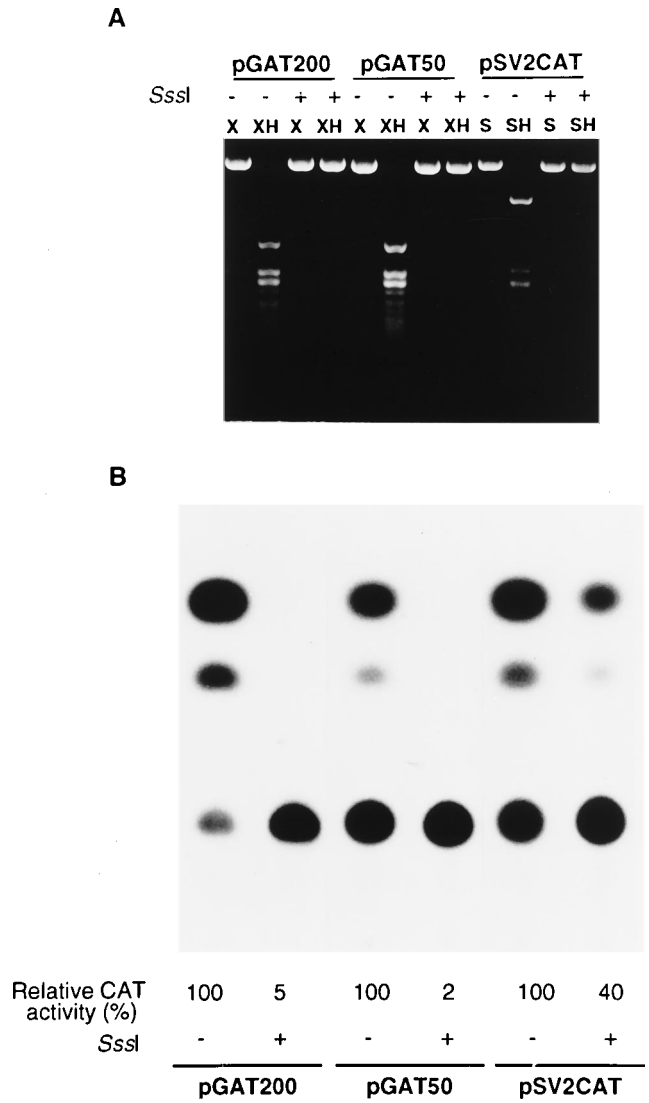

FIG. 5. Effect of in vitro methylation of the galectin-1 promoter region on the transcriptional activity. (A) pGAT200, pGAT50, and pSV2CAT were methylated with *Sss*I methylase. Both *Sss*I-treated (+) and -untreated (−) plasmids DNA were linearized with *Xba*I (X) or *Stu*I (S) and challenged with the methylation-sensitive restriction endonuclease *Hpa*II (H) to test for completeness of methylation. (B) Methylated plasmids were transfected in NIH 3T3 cells. Autoradiograms of representative CAT assays are shown. Relative CAT activities compared with that of each unmethylated construct are given. The values are the averages of three independent experiments.

DNA methylation during B-cell differentiation and activation (31). The CD43 (30) and galectin-1 (45) gene promoters show many features in common: (i) the region from −50 to +50 is sufficient to drive efficient transcription; (ii) Sp1 sites located about 60 bp upstream from the transcription initiation site can enhance promoter activity but are not necessary for basal transcription; (iii) in the promoter region, there are several stretches of sequence identities and a similar number of CpG dinucleotides; and (iv) both promoters contain (in the region from positions −3 to +7) a consensus sequence for an initiator element. Moreover, the interaction between the two gene products, CD43 and galectin-1, is crucial for the maturation of the T-lymphoblastoid cells (5). It will be of interest to study whether changes in DNA methylation could determine a coordinate expression of the two genes during these processes.

Since hypomethylation may be one of the crucial factors determining the progress of a tumor cell to a frank malignancy (15) and galectin-1 may play a role during carcinogenesis and in the acquisition of the metastatic phenotype (9, 27, 50, 52), it will be also interesting to extend the observations presented in this study to other conditions in which modulation of galectin-1 gene expression occurs, such as activation in human tumors (9) and differentiating agent-dependent repression (7, 33).

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