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GENE EXPRESSION

Developmental changes in the methylation status of regulatory elements in the murine α1(I) collagen gene

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Regulatory elements contributing to the tissue-specific regulation of the murine α1(I) collagen (Collal) gene have previously been identified in its promoter region and first intron. Because several lines of evidence indicate that DNA methylation may be involved in the tissue-specific regulation of Collal gene expression, we have analyzed the methylation status of the 5′ region of the gene by restriction analysis and a methylation-dependent PCR assay. All sites tested were unmethylated in sperm DNA. The region surrounding the start site of transcription was partially or completely methylated in undifferentiated embryonal cell lines, suggesting that it may be marked by de novo methylation during early embryonic development. In differentiated cells and adult tissues, the Collal promoter was completely demethylated in collagen-producing and some nonproducing cells, and partially methylated in other nonproducing cells. The first intron was unmethylated in collagen-producing as well as nonproducing cells. Only sites in the first exon showed an inverse correlation with transcriptional activity, i.e., they were unmethylated in cells that express the gene, but predominantly methylated in cells that do not. Our results indicate that the methylation status of a small area (<1 kb) downstream of the Collal promoter, but not of the promoter itself or the first intron, may be critical for transcriptional activity of the promoter, presumably through an indirect mechanism.

DNA methylation of the cytosine of CpG dinucleotides plays an important role in the regulation of gene expression and differentiation in vertebrates (Razin et al., 1984; Cedar 1988; Weissbach et al., 1989; Adams, 1990), and complex changes in the methylation pattern of mammalian genes can be observed during development (Monk et al., 1987; Monk, 1988). Tissue-specific genes usually show an inverse correlation between their methylation status and transcriptional activity; they are hypomethylated in tissues in which they are expressed and hypermethylated in tissues in which they are not expressed. The methylation status of promoter sequences has been shown to be especially critical for transcriptional activity (Doerfler et al., 1989). However, in many cases the expression of tissue-specific genes does not appear to correlate strictly with their methylation status (McKeon et al., 1982; Burch and Weintraub, 1983; Tanaka et al., 1983; Gautsch and Wilson, 1983; Enver et al., 1988). This may suggest that DNA methylation is not involved in the regulation of these genes or is a secondary event. Alternatively, methylation at only one or a few critical sites may be decisive for transcriptional activity, and this may not be easily detectable by the most commonly used experi-

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mental approach, the analysis with methylation-sensitive restriction enzymes.

The murine and human α1 type I collagen (Col1a1) genes contain two blocks of cis-regulatory elements located in the 5′ flanking region and the first intron that contribute to their transcriptional regulation (Rossouw et al., 1987; Bornstein et al., 1987; Rippe et al., 1989; Sherwood and Bornstein, 1990; Liska et al., 1990). Both blocks appear to be modular arrangements of positive as well as negative elements. DNase protection and mobility shift assays have identified various sequences in the promoter and first intron that interact with DNA-binding proteins (Brenner et al., 1989, Karsenty and de Crombrugghe, 1990; Liska et al., 1990; Nehls et al., 1991). Because several observations suggest that DNA methylation may be involved in the regulation of various collagen genes (Jähner and Jaenisch, 1985; Smith and Marsilio, 1988; Burbelo et al., 1990; Chan et al., 1991; Thompson et al., 1991), we have determined the methylation status of the murine Col1a1 gene regulatory elements in various collagen-producing and nonproducing cells and tissues. Our results show that, in contrast to most mammalian genes analyzed so far, the 5′ end of the Col1a1 gene is unmethylated in sperm DNA, confirming and extending previous observations (Jähner and Jaenisch, 1985). The region surrounding the start site of transcription appears to be marked by de novo methylation in early embryonic cells, and is subject to subtle changes in its methylation status during development. Interestingly, the methylation status of a region downstream of the start site of transcription, and not of the promoter itself or the first intron, is inversely correlated with transcriptional activity of the promoter.

Materials and methods

Cell lines

NIH 3T3 mouse fibroblasts, WEHI-3B mouse myelomonocytic leukemia cells (Metcalf and Nicola, 1982, provided by W. Ostertag), EL4 mouse lymphoma cells (ATCC, provided by M. Kagnoff), mouse hepatoma cells (Darlington et al., 1980, provided by G. Darlington), and mouse osteoblasts (Schmidt et al., 1989, provided by J. Schmidt) were grown in modified Eagle's medium with 10% iron-supplemented calf serum. P19 embryonal carcinoma cells (McBurney and Rogers, 1982, provided by E. Adamson) were grown in alpha-MEM supplemented with 10% fetal calf serum. DNA from D3 embryonic stem cells (Gossler et al., 1986) was kindly provided by H. Baribault.

Southern blot hybridizations and RNase protection assays

Restriction digestion and Southern blot hybridization analyses of genomic DNA from tissue culture cells and mouse tissues were performed as described previously (Breindl et al., 1984; Chan et al., 1991). The indicated probes from the Col1a1 gene were cloned into the pSP6/T7-19 plasmid (BRL) and transcribed into riboprobes in vitro (Chan et al., 1991). Hybridization and washing conditions were as described by Church and Gilbert (1984). RNase protection assays were performed essentially as described before (Chan et al., 1991).

DNA sequences

The DNA sequence referred to in this paper is in the EMBL Data Library (accession number X54876) and will be published elsewhere.

Results

Methylation of transcription factor binding sites in the Col1a1 promoter does not correlate with transcription

In order to study a possible correlation between Col1a1 gene expression and DNA methylation,

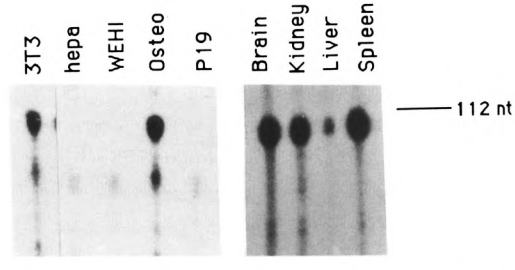
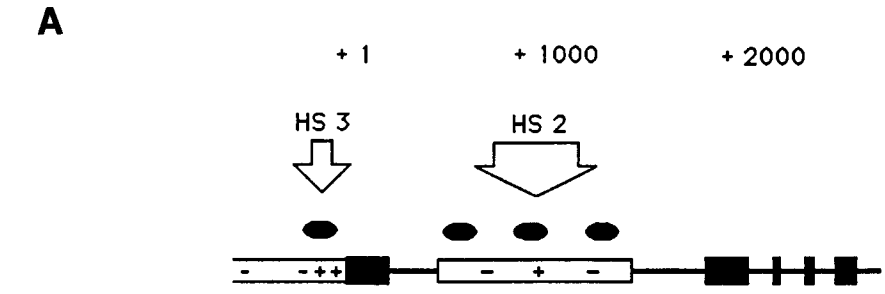
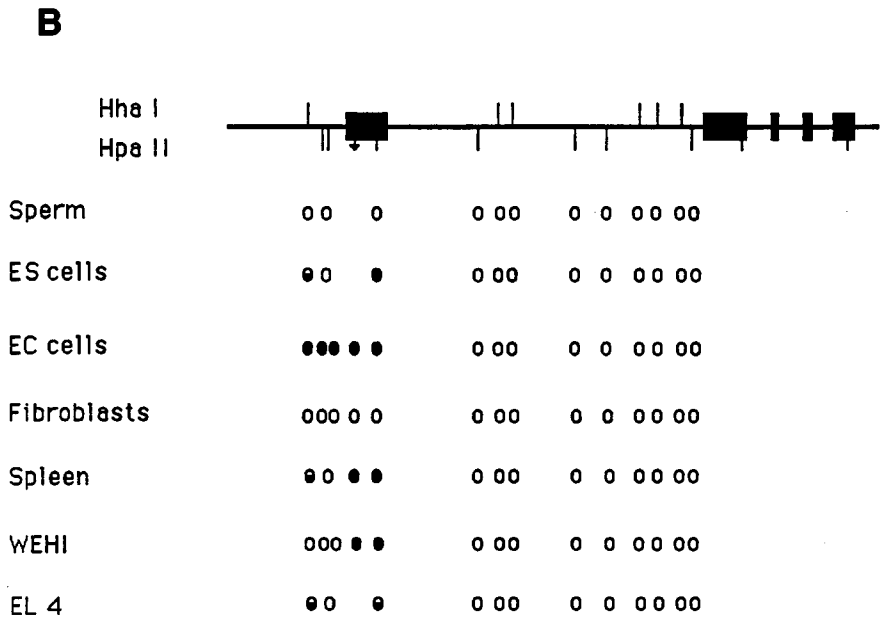


Figure 1. Col1a1 gene expression in murine cell lines and tissues. RNA was isolated from the indicated cell lines and tissues and analyzed by RNase protection assay as described (Chan et al., 1991) using a probe from the Col1a1 first exon that protects 112 nucleotides of Col1a1 mRNA. Ten μg total RNA from the indicated cell lines and 40 μg total RNA from the indicated tissues were used. The autoradiogram on the left was exposed overnight, and the one on the right for four days.





C

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-240 ACAGCACCTCTGGCCCATGTAGATCTGGGGGGCAAGGGCGGCAGAGTTGCGGGAGGGGGGGGCGCTGGGTGGACTCCTTTC
                                                          4    HhaI

-160 CCTTCCTTTCCCCTCCTCCCCCCTCTTCGTTCCAAATTGGGGGCCGGGCCAGGCAGTTCTGATTGGCTGGGGGCCGGGCTG
                      3                    2   HpaII                  1      HpaII

 -80 CTGGCTCCCCCTCTCCGAGAGGCAGGGTTCCTCCCAGCTCTCCATCAAGATGGTATAAAAGGGGCCCAGCCAGTCGTCGG

  +1 AGCAGACGGGAGTTTCTCCTCGGGACGGAGCAGGAGGCACGCGGAGTGAGGCCACGCATGAGCCGAAGCTAACCCCCCAC
                                          ThaI

 +81 CCCAGCCGCAAAGAGTCTACATGTCTAGGGTCTAGACATGTTCAGCTTTGTGGACCTCCGGCTCCTGCTCCTCTTAGGGG
                                                        HpaII

+161 CCACTGCCCTCCTGACGCATGGCCAAGAAGACA/GTAAGTT
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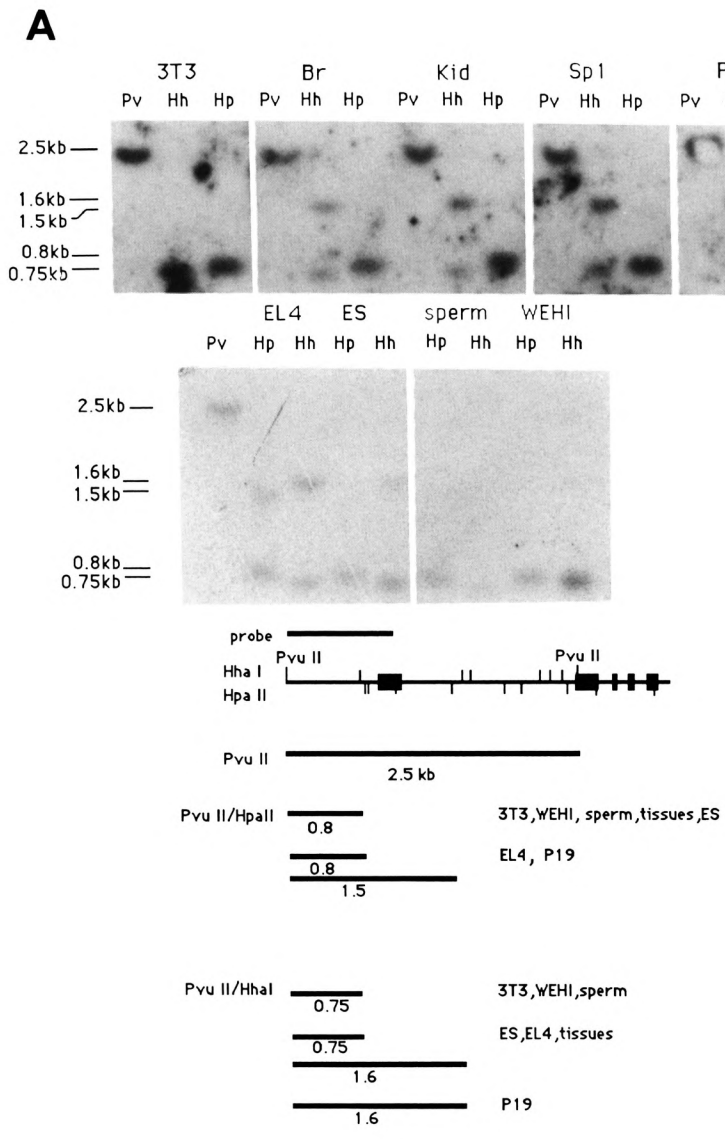
Figure 2. **A.** Summary of regulatory elements and potential factor-binding sites in the 5′ region of the murine Col1a1 gene. The number of nucleotides relative to the transcription start site (+1) is shown above the map. Filled boxes represent exons, and open boxes show the regions where positive (+) or negative (−) regulatory elements have been located. Filled ovals above the map indicate the location of potential factor-binding sites identified by DNaseI footprints and gel shift experiments. Arrows point to previously mapped DNase hypersensitive sites (for details, see the text). **B.** Location of restriction sites for Hpa II, Hha I, and Tha I (↓) in the 5′ region of the murine Col1a1 gene and summary of their methylation status in different cell types. ○, unmethylated; ●, methylated; ◒, partially methylated. **C.** Nucleotide sequence of the Col1a1 promoter and first exon (Harbers et al., 1984). The TATAA box, the footprints 1–4 referred to in the text, and restriction sites for the methylation-sensitive enzymes Hpa II, Hha I, and Tha I are underlined. The start site of transcription is +1, and the translation start codon ATG is underlined. The / between nucleotides 193 and 194 shows the beginning of the first intron.



we first analyzed by RNase protection analysis the expression of Col1a1 mRNA in various murine cell lines and tissues. The gene was abundantly transcribed in fibroblasts and osteoblasts, but not in WEHI-3B leukemia cells, mouse hepatoma cells, or the embryonal carcinoma (EC) cell line P19 (Fig. 1; Hartung et al., 1986). The Col1a1 gene was also transcribed in all major organs analyzed (Fig. 1). Expression was approximately 10-fold lower in brain, kidney, and spleen and approximately 100-fold lower in liver than in fibroblasts.

We have previously analyzed regulatory elements in the 5′ flanking region and the first intron of the Col1a1 gene (Rippe et al., 1989). The proximal 5′ regulatory elements located within 222 bp upstream from the start site of transcription exert a strong positive effect on Col1a1 promoter activity and are sufficient for its tissue-specific expression. Four protein-binding sites were identified in this region by footprint and gel shift analyses (Brenner et al., 1989; Karsenty and de Crombrugghe, 1990; Nehls et al., 1991; Fig. 2). Two of these (footprints 1 and 2) contain inverted CCAAT motifs and perfect 12 bp direct repeats TGGGGGCCGGGC which are conserved in the Col1a1 genes of other species (Chu et al., 1985; Lichtler et al., 1989); footprint 3 (CCTCCTCCCCCCTCTTC) is part of a polypyrimidine stretch which coincides with a pre-

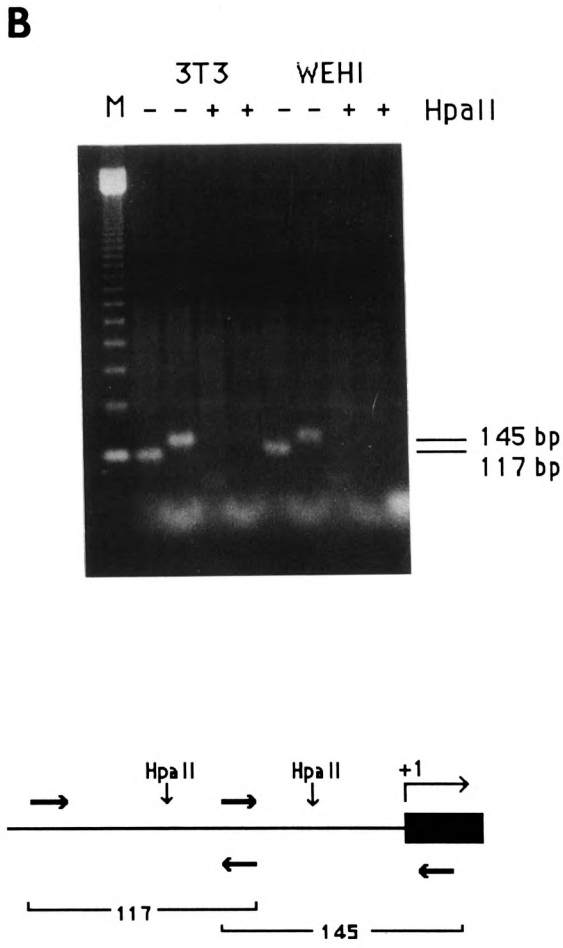


Figure 3. A. Methylation status of the regulatory elements in the Col1a1 promoter. Genomic DNA from the indicated cell lines and tissues was analyzed by restriction digestion and Southern blot hybridization. Digestion with Pvu II creates a 2.5 kb fragment when hybridized to the indicated probe. Further digestion with Hpa II or Hha I yields the fragments shown in the diagram, depending on the methylation status of the sites. **B.** Methylation status of closely adjacent Hpa II sites in the Col1a1 promoter analyzed by methylation-dependent PCR assay. Genomic DNA from 3T3 and WEHI-3B cells was digested with Hpa II and amplified by PCR as described in Materials and Methods. In uncut DNA, fragments of 117 bp and 145 bp, respectively, were amplified when primer pairs flanking the Hpa II sites were used. Amplification was completely abolished by prior digestion of the DNA with Hpa II, indicating that both Hpa II sites were unmethylated in both cell types.

viously mapped transcription-associated DNase-hypersensitive site (Breindl et al., 1984); and footprint 4 (GGGAGGGGGGGCGCTGGG) presumably interacts with a negative regulatory factor. Fortuitously, the only methylatable CpG dinucleotides in footprints 1 and 2 are part of Hpa II restriction sites; the only CpG in footprint 4 is part of a Hha I site; and footprint 3 contains no CpG (Fig. 2). Thus, restriction analysis with Hpa II and Hha I directly reveals the methylation status of binding sites for factors involved in the tissue-specific regulation of the Col1a1 gene.

Southern blot analyses demonstrated different methylation patterns of the Col1a1 gene 5′ proximal regulatory elements in collagen-producing and nonproducing cells, but no strict correlation of their methylation status with gene expression (Figs. 3A and 4, summarized in Fig. 2B). Digestion of genomic DNA from sperm, ES cells, 3T3 cells, WEHI cells, and various mouse tissues with Pvu II and Hpa II resulted in an 0.8 kb fragment when probed with the probe indicated in Figure 3A, indicating that either one or both of the Hpa II sites in footprints 1 and 2 were completely unmethylated in collagen-producing cells, as well as some nonproducing cells (also see below and Fig. 3B). Hpa II digestion of DNA from EL4 cells and P19 EC cells resulted in 0.8 kb and 1.5 kb fragments (Fig. 3A), indicating that the Hpa II sites in footprints 1 and 2 were partially methylated in these nonproducing cells. (We have obtained different results using different P19 cell clones; in two other clones the Hpa II sites in the promoter were completely methylated.) These data also indicate that a Hpa II site in the first exon was methylated in these cells (see below). Note that no larger fragments were observed; this excluded the possibility that the 1.5 kb fragments were due to incomplete restriction digestion, and showed that the next Hpa II site in the first

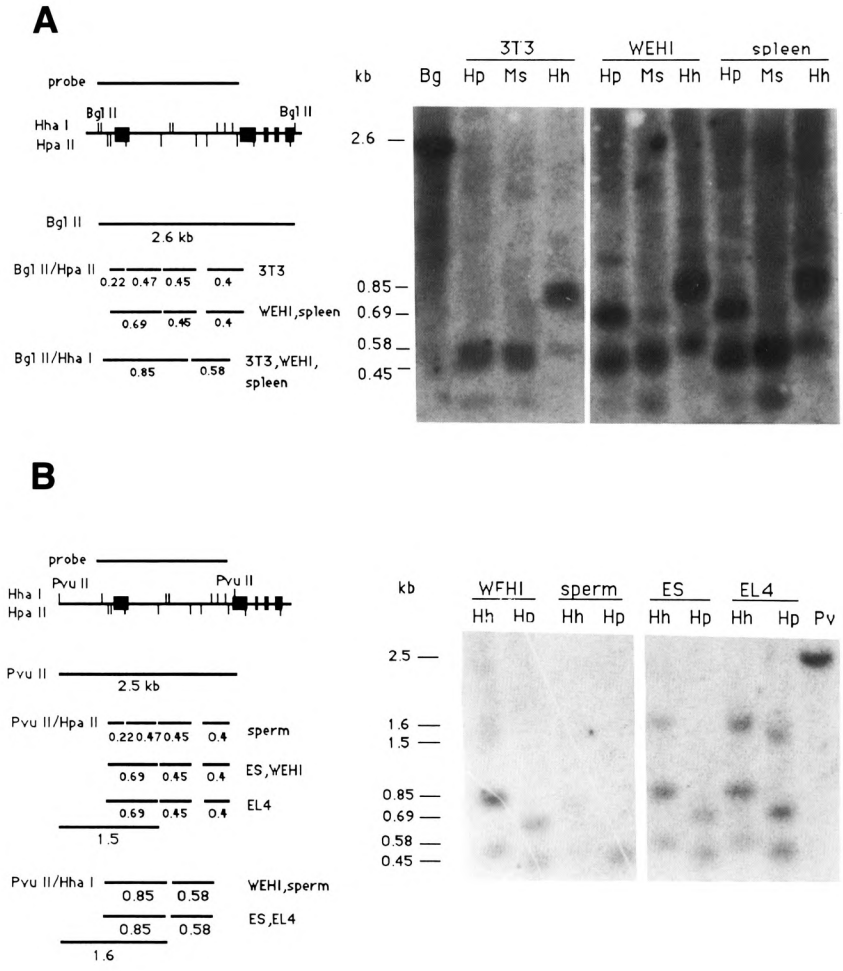


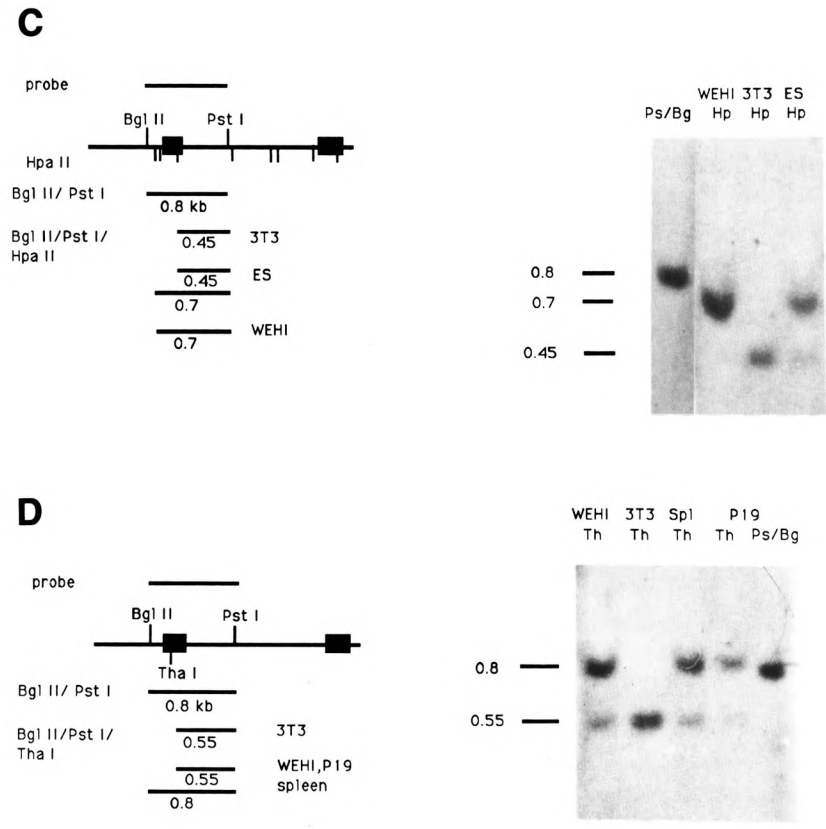
Figure 4. Methylation status of Hpa II and Hha I sites in the first exon and intron of the murine Col1a1 gene. Genomic DNA from the indicated cell lines and tissues was digested with Bgl II (**A**), which creates a 2.6 kb fragment, Pvu II (**B**), which creates a 2.5 kb fragment, or Pst I and Bgl II (**C** and **D**), which create a 0.8 kb fragment when hybridized with the indicated probes. Further digestion with Hpa II and Hha I (**A**, **B**, and **C**) or Tha I (**D**) yields the fragments shown in the diagrams, depending on the methylation status of the restriction sites.

intron was completely unmethylated in these cells.

Because the two Hpa II sites in footprints 1 and 2 are only 30 bp apart, the Southern blot analysis did not reveal whether one or both of them were unmethylated in cells in which this region was sensitive to Hpa II digestion. We have therefore used a methylation-dependent PCR assay to analyze the methylation status of both sites in collagen-producing 3T3 and nonproducing WEHI-3B cells. The results in Figure 3B show that amplification of genomic DNA from these cell types using primer pairs flanking each of the Hpa II sites was completely abolished by prior digestion with Hpa II, indicating that both sites are unmethylated in both cell types.

To determine the methylation status of footprint 4, genomic DNA was digested with Pvu II and Hha I. This resulted in a 0.75 kb fragment in DNA from sperm, 3T3 cells, and WEHI-3B cells (Fig. 3A), indicating that footprint 4 was unmethylated in these cells. In contrast, in DNA from ES cells, EL4 cells, and all tissues analyzed, it was partially methylated, generating 0.75 and 1.6 kb fragments. In P19 EC cells it was completely methylated, generating only the 1.6 kb fragment (Fig. 3A). Again, no larger fragments were observed, excluding incomplete restriction digestion and showing that the next Hha I site in the intron was unmethylated in all cells and tissues.

These results show that the regulatory elements in the Col1a1 promoter region are completely unmethylated in collagen-producing and



some nonproducing cells, and partially or completely methylated in other nonproducing cells, i.e., methylation of the promoter region is not correlated with transcriptional activity of the gene.

The first intron of the Col1a1 gene is unmethylated in collagen-producing and nonproducing cells

Regulatory elements located in the first intron of the Col1a1 gene have previously been identified in several laboratories (Rossouw et al., 1987; Bornstein et al., 1987; Rippe et al., 1989; Sherwood and Bornstein, 1990; Liska et al., 1990) but are less well characterized than the proximal 5′ elements. Several protein-binding sites were identified by footprint experiments (Fig. 2). An AP1 binding site was found in the first intron of the human and rat Col1a1 genes (Liska et al., 1990) and, because its sequence is completely conserved between nucleotides +542 and +573 in the murine gene (unpublished sequence data), is likely to also be present in the murine gene. A second binding site is located between nucleotides +900 and +1000 (unpublished observation); this region of the intron contains two enhancer core consensus sequences GTGGTTG/A and a DNase-hypersensitive site (Breindl et al., 1984). A third protein-binding site containing two adjacent Sp1 motifs is centered around nucleotide +1175 (unpublished observation).

An analysis of the methylation status of the intron regulatory elements is shown in Figure 4. Digestion of 3T3 cell DNA with Hpa II and Msp I resulted in an identical pattern of fragments (0.4, 0.45, and 0.47 kb, Fig. 4A; the smaller fragments generated by these enzymes are not visible), indicating that in collagen-producing cells all Hpa II sites in this region were unmethylated. The Hpa II sites were also completely unmethylated in sperm DNA (Fig. 4B), confirming earlier results (Jähner and Jaenisch, 1985). In contrast, Hpa II digestion of DNA from nonproducing WEHI-3B cells and total spleen resulted in fragments of <0.5 kb and an additional fragment of 0.7 kb (Fig. 4A). This indicates

that in these cells the Hpa II sites located in the first intron were unmethylated, whereas a site in the first exon was methylated (see the next paragraph).

Hha I digestion of all DNAs analyzed (3T3, WEHI-3B, spleen, sperm, ES, and EL4 cells) produced fragments of 0.85 and 0.58 kb (Fig. 4A and B; the smaller fragments produced by Hha I are not visible). This shows that all Hha I sites in the intron were unmethylated in all cells and tissues tested. The 1.6 kb bands in ES and EL4 cell DNAs (Fig. 4B) are due to the partial methylation of the Hha I site in the promoter region in these cells described above (Fig. 3). These results indicate that the regulatory elements in the first intron of the Col1a1 gene are unmethylated in both collagen-producing and nonproducing cells.

A region in the first exon of the Col1a1 gene is methylated differently in collagen-producing and nonproducing cells

The size of the additional fragment produced by Hpa II digestion of WEHI-3B and spleen DNA (Fig. 4A) suggested that a Hpa II site in the first exon of the gene was methylated in these cells. The same site was found to be completely methylated in P19 EC cells and partially methylated in EL4 cells (compare Fig. 3A). Additional experiments showed that the 0.7 kb Hpa II fragment was also produced in DNA from ES cells and EL4 cells, but not from sperm (Fig. 4B; the 1.5 kb fragment in EL4 cells results from the partial methylation of the Hpa II sites in the promoter in these cells described above, Fig. 3). Direct analysis confirmed that the Hpa II site in question is the one located in the first exon of the Col1a1 gene (Fig. 4C). Digestion of genomic DNA with Pst I and Bgl II produced a fragment of 0.8 kb when probed with the indicated probe. Further digestion with Hpa II produced a fragment of 0.45 kb with 3T3 cell DNA (Fig. 4C; smaller fragments of 0.27, 0.1, and 0.03 produced by Hpa II are not visible). In contrast, with WEHI-3B and ES cell DNAs, a 0.7 kb fragment was produced (Fig. 4C; with ES cell DNA a small amount of the 0.45 kb fragment was also seen). This confirmed that the Hpa II site in the first exon was unmethylated in collagen-producing cells, but completely or predominantly methylated in nonproducing cells. This site was also unmethylated in sperm DNA (not shown).

We also determined the methylation status of a Tha I restriction site located further upstream in the first exon and found that this site was completely unmethylated in collagen-producing 3T3 cells, whereas it was predominantly methylated in nonproducing cells (P19, WEHI-3B) and spleen DNA (Fig. 4D). These results show that a small region downstream of the promoter in the first exon of the Col1a1 gene is unmethylated in collagen-producing cells but predominantly methylated in nonproducing cells (except sperm), indicating that methylation of this region may be critical for the activity of the Col1a1 promoter. It is worth noting, though, that methylation of the tested restriction sites in this region usually was partial and not complete (Fig. 4D; and additional data not shown). A schematic summary of the methylation analysis is shown in Figure 2B.

Discussion

The developmental activation and regulation of genes in mammals is associated with complex changes in their methylation patterns, and frequently such changes are restricted to promoter regions or other regulatory DNA sequences. Overall, the murine genome is relatively highly methylated in sperm and undermethylated in the egg (Monk, 1988). During preimplantation development, a loss of overall methylation occurs, followed by progressive de novo methylation in extraembryonic and embryonic tissues (Monk et al., 1987; Monk, 1988). In differentiated tissues, most tissue-specific genes show an inverse correlation between expression and methylation: they are hypermethylated in tissues in which they are transcriptionally inactive and hypomethylated in tissues in which they are expressed (Ysraeli and Szif, 1984; Cedar, 1988; Weissbach et al., 1989).

The methylation status of regulatory elements in the 5′ flanking region and the first intron of the murine Col1a1 gene showed some deviations from the above rules (Figs. 2B, 3, and 4). Most notably, the Col1a1 5′ region was found to be completely unmethylated in sperm DNA at all sites analyzed, confirming and extending previous observations (Jähner and Jaenisch, 1985). This is in contrast to the rule that genes are usually most highly methylated in sperm DNA (Ysraeli and Szif, 1984; Monk, 1988). Lack of methylation in sperm DNA has been correlated

with the presence of CpG-rich islands in many housekeeping and tissue-specific genes (Bird, 1986; Gardiner-Garden and Frommer, 1987). However, the murine Col1a1 gene 5′ region is unmethylated in sperm DNA although it has no CpG-rich islands (unpublished sequence data). More likely, as noted by Groudine and Conklin (1985), sites of undermethylation in sperm DNA may correspond to the locations of DNase-hypersensitive chromatin sites in somatic tissues and function as signals for gene activation early in development. For the Col1a1 gene, both correlations apply: several DNase-hypersensitive sites have been mapped in this region of the gene (Breindl et al., 1984), and expression of type I collagen has been observed as early as day 8 of embryonic development, both in embryonic and extraembryonic tissues (Adamson and Ayers, 1979; Leivo et al., 1980).

Mouse embryonic stem (ES) cells (Evans and Kaufman, 1981) and embryonal carcinoma (EC) cells (Martin, 1980; McBurney and Rogers, 1982) were used to analyze the methylation status of Col1a1 regulatory sequences in early embryonic cells. In both cell types the region surrounding the start site of transcription of the Col1a1 gene was found to be partially or completely methylated at all sites tested (Figs. 2B, 3, and 4). We do not think that this is an artificial de novo methylation, as has been observed in cell lines after prolonged maintenance in tissue culture (Antequera et al., 1990), because the methylation of the Col1a1 5′ region in ES and EC cells was restricted to a defined area and did not include regulatory sequences further downstream in the first intron, and because we did not observe a comparable de novo methylation in other cell lines such as WEHI-3B, in which the Col1a1 promoter was completely unmethylated (Figs. 2B, 3, and 4). Furthermore, D3 ES cells are undifferentiated and contribute to the germ line with high frequency when introduced into transgenic mice (Gossler et al., 1986). Similarly, P19 EC cells can be induced to differentiate under appropriate conditions into nerve cells, fibroblast-like cells, and skeletal and heart muscle cells (McBurney et al., 1982). Thus, both cell lines appear to resemble genuine early embryonic cells, and we therefore think that the differences in the methylation patterns in sperm, ES cells, and EC cells reflect a de novo methylation of the region surrounding the Col1a1 promoter in early mouse development, as has been observed for other sequences (Monk et al., 1987; Monk, 1988). This will have to be verified by analyzing the methylation pattern of this region in early embryonic tissues. If confirmed, this observation would indicate that the de novo methylation of genes during early embryonic development may not be "global." At least for some genes, it may be restricted to key regulatory regions that are "marked" by de novo methylation in early embryogenesis and that maintain a differential methylation pattern and gene activity throughout development.

In differentiated cells, we found no correlation between Col1a1 gene expression and the methylation status of the previously identified regulatory elements in the Col1a1 promoter and first intron. The only consistent difference in the methylation pattern between collagen-producing and nonproducing cells was in the first exon of the gene, i.e., the same region that was de novo methylated in ES and EC cells (Figs. 2B, 3, and 4). This region was unmethylated in cells that express the Col1a1 gene but predominantly methylated in cells that do not (with the exception of sperm, which presumably does not synthesize collagen but was unmethylated). These results indicate that methylation of a rather small area (<1 kb) downstream of the Col1a1 promoter, and not of the promoter itself, may be critical for determining the transcriptional activity of that promoter. They also show that the differences in DNA methylation of a transcriptionally active and inactive version of a gene can be very subtle (only two of the analyzed restriction sites were differently methylated in 3T3 and WEHI-3B cells, Fig. 2B) and in some cases may not be detectable at all by conventional restriction analysis. It is obvious that very precise mechanisms must exist to establish and maintain such methylation patterns in development. This is in agreement with a recently proposed model which postulates a high efficiency in maintenance methylation, and indicates that the methylation status of individual CpG sites is maintained autonomously (Pfeifer et al., 1990).

By what mechanism might methylation of the region downstream of the Col1a1 promoter be involved in the repression of Col1a1 transcription in WEHI-3B cells and possibly other nonproducing cells? DNA methylation presumably alters DNA-protein interactions and most likely interferes with gene expression in at least

two different ways, by directly inhibiting the binding of transcription factors (Iguchi-Ariga and Schaffner, 1988; Watt and Molloy, 1988; Predergast and Ziff, 1991), and more indirectly by interfering with the assembly of a transcription-competent chromatin structure (Weintraub, 1985; Keshet et al., 1986; Buschhausen et al., 1987), which may be mediated by a methyl-CpG binding protein (Boyes and Bird, 1991; Levine et al., 1991). So far our data do not allow us to determine which of these two mechanisms is involved in Coll a1 gene repression. We have obtained preliminary evidence that a protein-binding site is located within the region that is differentially methylated in collagen-producing and nonproducing cells at the 3′ end of the first exon (unpublished observation). It is possible that the factor(s) binding to this site is decisive for Coll a1 promoter activity and sensitive to DNA methylation. It may be relevant in this context that in the mutant Mov13 mouse strain a retroviral provirus has integrated in close vicinity of this site and inactivates expression of the Coll a1 gene in a tissue-specific manner at the level of transcriptional initiation (Harbers et al., 1984; Hartung et al., 1986; Kratochwil et al., 1989), lending support to the assumption that this region is of crucial importance for Coll a1 promoter activity.

It is more likely that methylation of the region downstream of the promoter contributes to transcriptional suppression of the Coll a1 promoter by an indirect mechanism, e.g., an alteration of the local chromatin structure that silences the gene by rendering the promoter inaccessible to trans-acting factors. The observations that the factors interacting with the Coll a1 promoter are ubiquitous factors and are abundant in cells that do not express the Coll a1 gene, that none of the factors appears to be inhibited by DNA methylation to bind to its target DNA (Nehls et al., 1991; and unpublished results), and that a DNase-hypersensitive chromatin structure in the Coll a1 promoter region is present only in collagen-expressing cells (Breindl et al., 1984) favor this notion. An indirect mechanism may also explain why the Hpa II and Hha I sites downstream of the Coll a1 promoter are often partially—and not completely—methylated in cells that do not produce collagen (Fig. 4C and D), because such a mechanism may not require a precise methylation pattern but rather a certain number or density of methylated CpGs.

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