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DNA Methylation Represses the Murine α1(I) Collagen Promoter by an Indirect Mechanism

KATHERINE RHODES,[1] RICHARD A. RIPPE,[2]† AKIHIRO UMEZAWA,[2] MICHAEL NEHLS,[2] DAVID A. BRENNER,[2]† AND MICHAEL BREINDL[1]*

Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, California 92182,[1] and Department of Medicine and Program in Molecular Pathology, University of California and Veterans Administration, San Diego, California 92093[2]

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Several lines of evidence indicate that DNA methylation plays a role in the transcriptional regulation of the murine α1(I) collagen gene. To study the molecular mechanisms involved, a reporter gene construct containing the α1(I) promoter and part of the first exon linked to the luciferase gene (Col3luc) was methylated in vitro and transfected into murine fibroblasts and embryonal carcinoma cells. Methylation resulted in repression of the α1(I) promoter in both cell types, although it was less pronounced in embryonal carcinoma cells than in fibroblasts. The extent of repression depended on the density of methylation. DNase footprint and mobility shift assays indicated that the *trans*-acting factors binding to the α1(I) promoter and first exon are ubiquitous factors and that their DNA binding is not inhibited by methylation. Transfection of Col3luc into *Drosophila* SL2 cells together with expression vectors for the transcription factors Sp1 and NF-1 showed that DNA methylation also inhibits the α1(I) promoter in nonvertebrate cells, although to a much lesser extent than in murine cells. However, Sp1 and NF-1 transactivated the unmethylated and methylated reporter gene in SL2 cells equally well, confirming that these factors can bind and transactivate methylated DNA and indicating that DNA methylation represses the α1(I) promoter by an indirect mechanism. This was further confirmed by cotransfection experiments with unspecific methylated competitor DNA which partially restored the activity of the methylated α1(I) promoter. Our results suggest that DNA methylation can inhibit promoter activity by an indirect mechanism independent of methyl-C-binding proteins and that in vertebrate cells, chromatin structure and methyl-C-binding proteins cooperatively mediate the transcriptional inhibitory effect of DNA methylation.

DNA methylation at CpG dinucleotides is involved in fundamental biological processes in vertebrates such as the tissue-specific regulation of gene expression, genomic imprinting, and X chromosome inactivation (6, 15), and complex changes in the methylation patterns of mammalian genes take place during mammalian development (48, 49). The molecular mechanisms by which DNA methylation affects gene expression are only partly understood. DNA methylation presumably alters DNA-protein interactions and can interfere with gene expression in at least three different ways. First, it can directly affect the binding of transcription activators or repressors. Various sequence-specific DNA binding proteins that are inhibited by DNA methylation have been described. These include a factor binding to cyclic AMP-responsive elements (33), a HeLa cell factor stimulating the adenovirus major late promoter (64), the transcription factor AP-2 (19), the c-Myc, c-Myb, and v-Myb oncoproteins (40, 56), two transcription factors binding to the retinoblastoma gene promoter (53), NF-κB binding to the human immunodeficiency virus type 1 long terminal repeat (3), and proteins interacting with intracisternal A-particle long terminal repeat enhancers (25, 41). Other transcription factors, such as Sp1 and NF-1, were found to be insensitive to DNA methylation (4, 29, 31). Second, DNA methylation can affect the structure of chromatin. Evidence for this comes from experiments which show that transfected methylated DNA preferentially assumes a closed, transcriptionally inactive chromatin structure (39) and that chromatin structure formation is required to block transcription of microinjected methylated DNA (14). A recent study shows directly that histone H1, which is known to be a general transcriptional repressor (21, 65), shows binding to and transcriptional inhibition of methylated templates that is stronger than those of unmethylated templates (44), suggesting that the preferential binding of histone H1 to methylated DNA plays a role in the methylation-mediated inhibition of transcriptional activity. Third, DNA methylation can inhibit transcription through methyl-C-binding proteins, i.e., proteins which bind specifically to methylated but not unmethylated DNA. Several such proteins have been identified to date. Methyl-DNA binding protein 1 (MDBP-1) from human placenta binds to DNA in a sequence-specific manner dependent on the density of methylated CpGs (32). Methyl-C binding protein 1 (MeCP1) binds sequence independently to DNA that has at least 15 methylated CpGs (47). This protein inhibits the transcriptional activity of various promoters when they are methylated but not when they are unmethylated (8). A different protein, MeCP2, requires only a single mCpG for binding and inhibits transcription of both methylated and unmethylated DNA templates (46, 47). Another protein, MDBP-2, has been suggested to function as a repressor of the avian vitellogenin gene. This protein binds in a sequence-independent manner, requires a single methylated CpG for binding, and appears to be a member of the histone H1 family (35, 36, 55).

* Corresponding author. Mailing address: Department of Biology, LS426, San Diego State University, San Diego, CA 92182. Phone: (619) 594-2983. Fax: (619) 594-5676. Electronic mail address: mbreindl@sunstroke.sdsu.edu.

† Present address: Department of Medicine, University of North Carolina, Chapel Hill, NC 27599-7080.

TABLE 1. Sequence and origin of oligonucleotides

Oligonucleotide	Sequence	Origin	Reference
FP1	5′ CTAGCTGATTGGCTGGGGGCCGGGCT 3′ 3′ GACTAACCGACCCCCGGCCCGACTAG 5′	−103 to −82 in α1(I) promoter	Fig. 2
FP2	5′ AGCTTCCAAATTGGGGGCCGGGCCAG 3′ 3′ AGGTTTAACCCCCGGCCCGGTCCTAG 5′	−131 to −110 in α1(I) promoter	Fig. 2
FP3	5′ AGCTTCCCTCCTCCCCCCTCTTC 3′ 3′ AGGGAGGAGGGGGGAGAAGTCGA 5′	−152 to −134 in α1(I) promoter	Fig. 2
FP4	5′ AGCTGGGAGGGGGGGCGCTGGGT 3′ 3′ CCCTCCCCCCCGCGACCCATCGA 5′	−190 to −172 in α1(I) promoter	Fig. 2
EX1	5′ AGCTCACTGCCCTCCTGACGCATGGCCAAGAAGACA 3′ 3′ GTGACGGGAGGACTGCGTACCGGTTCTTCTGTGATC 5′	+162 to +193 in α1(I) first exon	Fig. 2
NF-1	5′ GTTTTGGATTGAAGCCAATATGAG 3′ 3′ AAAACCTAACTTCGGTTATACT 5′	Adenovirus origin of replication	18
AP-1	5′ TAAAGCATGAGTCAGACACCTC 3′ 3′ ATTTCGTACTCAGTCTGTGGAG 5′	Human collagenase promoter	1
Sp1	5′ GATCGGGGCGGGGC 3′ 3′ CCCCGCCCCGCTAG 5′	Simian virus 40 early promoter	23

Two mouse nuclear proteins have been described that bind selectively to a methylated c-Myc binding sequence (62). A sequence-independent methyl-C-binding protein (DBPm) has also been described for plants (24). The precise functions of most of these proteins remain to be elucidated. However, several studies have shown that methylation-mediated transcriptional repression can be reversed by excess unrelated, methylated DNA (8, 42). This suggests that binding of methyl-C-binding proteins to DNA may block *cis*-regulatory sequences and make them inaccessible to activating *trans*-acting factors.

We are studying the molecular mechanisms that regulate the stage- and tissue-specific expression of the murine α1 type I collagen gene (7). Several observations suggest that DNA methylation plays a role in the regulation of various collagen genes. In the mutant Mov13 mouse strain, transcriptional inactivation of the α1(I) collagen gene by insertion of a retroviral provirus (10, 30) is associated with changes in the methylation pattern of the cellular sequences (16, 34). In vitro methylation of the rat α2(I) promoter and the human α1(I) promoter abolishes their transcriptional activity (27, 63). The murine α1(IV) collagen gene is inactive in undifferentiated F9 teratocarcinoma cells and can be activated by demethylating agents (13, 17). Chemical transformation of rat liver epithelial cells results in transcriptional repression of the α2(I) collagen gene, which is associated with hypermethylation of the α2(I) promoter (61). A previous analysis of the murine α1(I) collagen gene has shown that regulatory elements in the 5′ region of the gene are subject to developmental changes of their methylation status and that the α1(I) promoter is unmethylated in collagen-producing cells, whereas it is partly or completely methylated in at least some nonproducing cells (57). In the present paper, we describe experiments which support the conclusion that DNA methylation represses α1(I) promoter activity by an indirect mechanism, presumably involving both assembly into inactive chromatin structure and interactions with a methyl-C-binding protein(s).

MATERIALS AND METHODS

Cell lines. NIH 3T3 mouse fibroblasts, WEHI 3B mouse myelomonocytic leukemia cells, mouse osteoblasts, mouse F9 and P19 embryonal carcinoma (EC) cells, and *Drosophila* Schneider L2 cells (kindly provided by J. Posakony) were grown as described before (51, 52, 57).

Preparation of nuclear extracts and DNase I protection and gel retardation assays. Nuclear proteins from NIH 3T3 cells, WEHI 3B cells, and P19 EC cells were extracted according to previously published methods (22, 60), and DNase I protection and gel retardation assays were performed as described elsewhere (52).

Transfection assays. Transfections were performed according to a standard calcium phosphate coprecipitation protocol, and β-galactosidase (β-Gal), luciferase, and chloramphenicol acetyltransferase (CAT) reporter gene assays were performed as described elsewhere (52). The amount of transfected DNA was kept constant by adding insertless plasmid DNA. All reporter gene assays were normalized for transfection efficiency by cotransfecting a constant amount of a β-Gal reporter gene and determining β-Gal activity.

Plasmids and oligonucleotides. pCol3luc containing the α1(I) promoter and part of the first exon (−220 to +116) and the luciferase reporter gene was described before (52). Construction of pRSVβ-gal containing the Rous sarcoma virus (RSV) promoter and the β-Gal reporter gene was described before (58). pBLCAT 19, which contains three AP2 binding sites, two Sp1 binding sites, and one NF-1 binding site driving the CAT reporter gene, was described before (66) and was a gift from R. Tjian. The *Drosophila* expression vectors pPacSP1 containing the *Drosophila* actin promoter and an Sp1 cDNA, pPacNF-1 containing the *Drosophila* actin promoter and an NF-1 cDNA, and pPadhAP2 containing the *Drosophila* adh promoter and an AP2 cDNA were described before (20, 66) and were also gifts from R. Tjian. The COPIA-LTR β-Gal plasmid (28) was kindly provided by S. Gray.

All oligonucleotides were synthesized with a Cyclone Plus oligonucleotide synthesizer (Milligen, Novato, Calif.) or an ABI Applied Biosystems 392 DNA synthesizer and were purified by thin-layer chromatography or high-performance liquid chromatography. The DNA sequences of the oligonucleotides used in the experiments are listed in Table 1.

In vitro DNA methylation. DNA was methylated in vitro by *Hha*I, *Hpa*II, or *Sss*I methylase according to the recommendations of the suppliers. *Micrococcus luteus* genomic DNA was sonicated to an average length of ~2 kb before methylation. The completeness of all methylation reactions was determined by restriction digestion with methylation-sensitive restriction enzymes, and the extent of methylation was always >90%. The central CpG in the EX1 oligonucleotide was methylated by replacing dCTP with methyl-dCTP in the corresponding cycle during oligonucleotide synthesis.

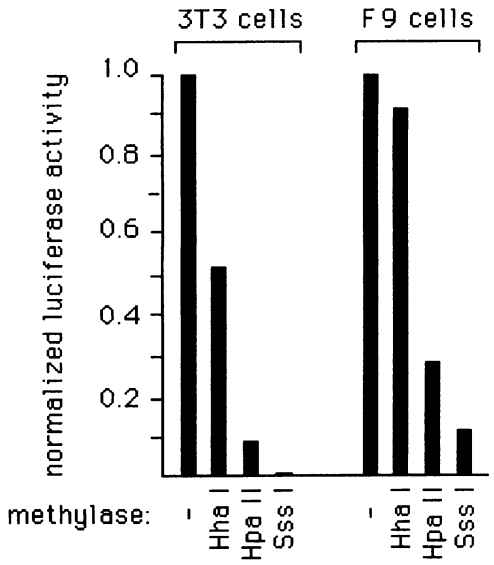

FIG. 1. In vitro methylation inhibits α1(I) promoter activity. Plasmid pCol3luc was methylated or mock methylated in vitro with *Hha*I, *Hpa*II, or *Sss*I methylase as indicated, and 2 μg was cotransfected with pRSVβ-gal into NIH 3T3 fibroblasts or F9 EC cells as indicated. Cells were harvested 48 h after transfection, and luciferase activity was determined and normalized for transfection efficiency (β-Gal activity).

Nucleotide sequence accession number. The DNA sequence referred to in this paper has been deposited in the EMBL data library under accession number X54876.

RESULTS

Transcriptional activity of the α1(I) promoter is inhibited by DNA methylation. We first performed transfection experiments with methylated and unmethylated pCol3luc, a reporter gene construct which contains the α1(I) promoter and the luciferase reporter gene (52), to determine whether the transcriptional activity of the murine α1(I) collagen promoter is sensitive to DNA methylation. Because studies by others (9) have shown that the extent of transcriptional repression by DNA methylation depends on the density of methyl-CpGs, we methylated the reporter gene with *Hha*I, *Hpa*II, or *Sss*I methylase. The first two enzymes methylate only their respective restriction sites, whereas the last enzyme methylates every CpG dinucleotide. The constructs were transfected transiently into NIH 3T3 cells and (for reasons discussed below) into F9 EC cells, and promoter activity was determined by measuring luciferase activity. All transfections were performed as cotransfections with pRSVβ-gal, which contains the β-Gal gene linked to the RSV promoter (58), and luciferase activity was normalized for transfection efficiency as determined by β-Gal activity. Figure 1 shows that methylation with *Hha*I methylase reduced α1(I) promoter activity in NIH 3T3 cells 2-fold, methylation with *Hpa*II methylase reduced α1(I) promoter activity 5- to 10-fold, and methylation with *Sss*I methylase reduced α1(I) promoter activity about 50-fold. Furthermore, methylation also inhibited α1(I) promoter activity in F9 EC cells, although to a lesser extent (Fig. 1). These results show that the murine α1(I) promoter is repressed by DNA methylation and that the extent of this repression depends on the density of methyl-CpGs.

Ubiquitous transcription factors interact with regulatory elements in the α1(I) collagen proximal promoter and first exon. To elucidate the mechanism by which DNA methylation represses α1(I) promoter activity, we first analyzed the transcription factors that interact with the α1(I) promoter. Previous experiments have shown that the proximal α1(I) promoter has four binding sites for transcription factors which are present in nuclear extracts from collagen-producing fibroblasts (11, 37, 51, 52) (Fig. 2). To explore whether different transcription factors which may be responsible for the cell-specific transcriptional activity of the α1(I) promoter are present in collagen-producing and nonproducing cells and/or whether binding of these factors is affected by DNA methylation, we performed DNase I footprint and mobility shift experiments with nuclear extracts from different cell types and used unmethylated and methylated oligonucleotides for the mobility shift assays. The collagen-producing cells used were NIH 3T3 fibroblasts, which produce relatively high levels of type I collagen and are unmethylated at all sites tested (57, 59). The nonproducing cell lines used were WEHI-3B myelomonocytic leukemia cells, in which the promoter is unmethylated but in which the first exon is methylated, and P19 EC cells, in which both the promoter and first exon are methylated (57).

Figure 2A shows that nuclear extracts from WEHI-3B cells produced the same footprints as NIH 3T3 nuclear extracts in the α1(I) promoter, indicating that identical or very similar factors capable of binding to the four previously identified factor binding sites in the α1(I) promoter are present in both cell types. Identical footprints were also produced by nuclear extracts from osteoblasts (data not shown). In contrast, a slightly different footprint pattern was observed with extracts from P19 EC cells (Fig. 2A). Footprints 1 and 4 (FP1 and FP4) appeared to be identical to those in NIH 3T3 cells, whereas FP3 was absent and FP2 covered a larger sequence. It is possible that the factor binding to the extended FP2 is involved in the transcriptional repression of the α1(I) promoter in P19 cells and/or that the FP3-binding factor is important for the developmental activation of the α1(I) promoter; however, these questions will have to be addressed by additional experiments. The localizations of the footprints in the α1(I) promoter and their DNA sequences are shown in Fig. 2B and C.

The promoter-binding factors present in NIH 3T3 cells and WEHI-3B cells were further compared by mobility shift assays. Oligonucleotides containing the four footprint sites (FP1 to FP4) showed identical mobility shifts with nuclear extracts from NIH 3T3 and WEHI-3B cells (Fig. 3). FP1 and FP2 have previously been shown to interact with the ubiquitous transcription factors Sp1 and NF-1 (51, 52), while FP3 and FP4 interact with a binding factor that has only recently been identified which also appears to be present in collagen-producing as well as nonproducing cells (36a). Thus, the transcription factors interacting with the proximal α1(I) collagen promoter appear to be ubiquitous and to be present in at least some cells in which the gene is not transcribed.

Since the first exon of the α1(I) collagen gene is differentially methylated in collagen-producing and nonproducing cells (57), we wanted to determine whether this region of the gene contains a *cis*-acting regulatory element that may be involved in determining the tissue-specific activity of the α1(I) promoter and whether it is sensitive to DNA methylation. A DNase footprint analysis (Fig. 4) showed that the 3′ end of the first exon does contain a factor-binding site. Inspection of the nucleotide sequence of this footprint (underlined in the sequence shown in Fig. 2C) revealed the presence of a consensus sequence for the transcription factor NF-1 (TGAC NNNNNGCCAAG) (45). We therefore performed mobility

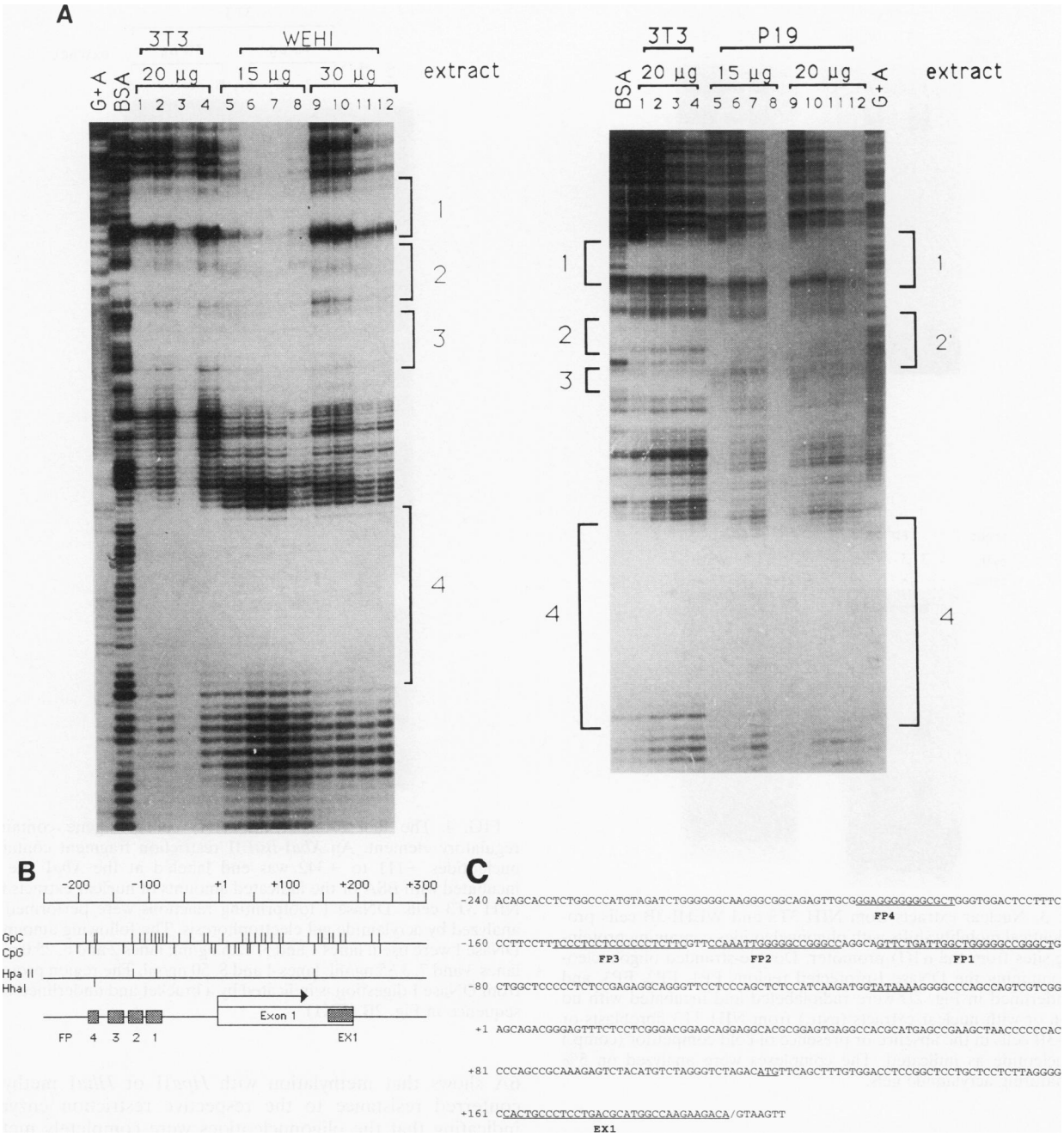

FIG. 2. DNase I protection assay of the α1(I) promoter region. (A) A *Bgl*II-*Xba*I restriction fragment containing the α1(I) promoter region (nucleotides −220 to +110) was end labeled at the *Bgl*II site. The probe was incubated with bovine serum albumin (BSA) or the indicated amounts of nuclear extracts from NIH 3T3 cells, WEHI-3B cells, or P19 cells as indicated. DNase I footprinting reactions were performed and analyzed by acrylamide gel electrophoresis. The following amounts of DNase I were used: lanes 1, 5, and 9, 0.5 ng/ml; lanes 2, 6, and 10, 1.0 ng/ml; lanes 3, 7, and 11, 1.5 ng/ml; lanes 4, 8, and 12, 2.0 ng/ml. Regions protected from DNase I digestion (footprints) are indicated by brackets. (B) Schematic map of the α1(I) promoter and first exon showing the distribution of GpC and CpG dinucleotides, *Hpa*II and *Hha*I restriction sites, and the locations of the transcription factor binding sites FP1 to FP4 and EX1 referred to in the text. (C) Nucleotide sequences of the proximal promoter and first exon of the murine α1(I) collagen gene. Regions protected from DNase I digestion in Fig. 2B, the TATAAA box, and the ATG translational initiation codon are underlined. The boundary between the first exon and intron is indicated by a shill.

shift assays with an oligonucleotide probe containing this sequence and various competitor oligonucleotides. Figure 5A shows that binding of the factor to the exon I oligonucleotide was outcompeted by excess cold homologous oligonucleotide or an oligonucleotide containing a consensus NF-1 binding site, but not by oligonucleotides containing AP-1 or Sp1 binding sites. This shows that the first exon of the α1(I) collagen gene contains a regulatory element which interacts with a member of the NF-1 family of transcription factors. Identical band shifts were obtained with this oligonucleotide

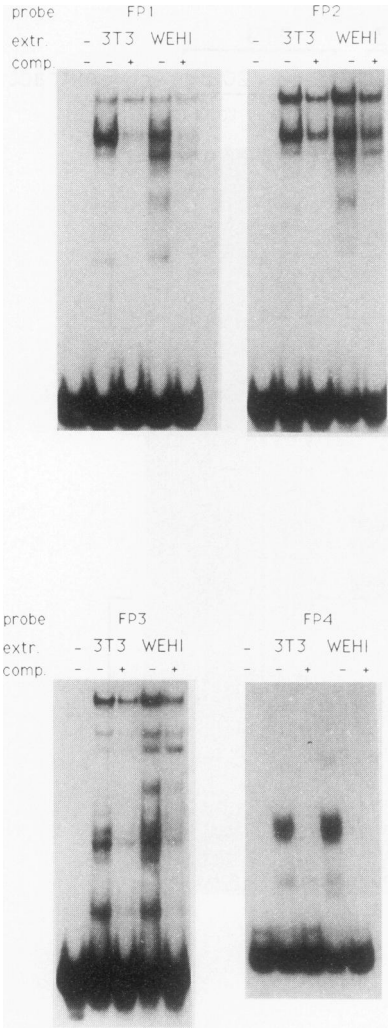

FIG. 3. Nuclear extracts from NIH 3T3 and WEHI-3B cells produce identical mobility shifts with oligonucleotides containing protein-binding sites from the α1(I) promoter. Double-stranded oligonucleotides containing the DNase I-protected regions FP1, FP2, FP3, and FP4 underlined in Fig. 2B were radiolabeled and incubated with no addition or with nuclear extracts (extr.) from NIH 3T3 fibroblasts or WEHI-3B cells in the absence or presence of cold competitor (comp.) oligonucleotide as indicated. The complexes were analyzed on 5% nondenaturing acrylamide gels.

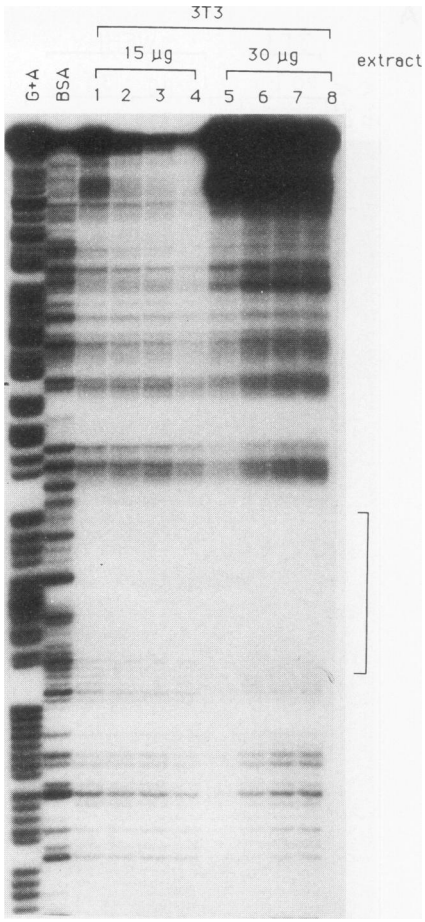

FIG. 4. The first exon of the α1(I) collagen gene contains a regulatory element. An *Xba*I-*Bst*EII restriction fragment containing nucleotides +111 to +342 was end labeled at the *Xba*I site and incubated with BSA or the indicated amounts of nuclear extracts from NIH 3T3 cells. DNase I footprinting reactions were performed and analyzed by acrylamide gel electrophoresis. The following amounts of DNase I were used: lanes 1 and 5, 12.5 ng/ml; lanes 2 and 6, 25.0 ng/ml; lanes 3 and 7, 37.5 ng/ml; lanes 4 and 8, 50 ng/ml. The region protected from DNase I digestion is indicated by a bracket and underlined in the sequence in Fig. 2B (EX1).

and WEHI-3B nuclear extracts (data not shown), indicating that the NF-1-like factor binding to the exon element is also a ubiquitous factor.

Binding of the transcription factors to the α1(I) collagen proximal promoter and first exon is not sensitive to DNA methylation. To determine the mechanism by which methylation represses the α1(I) collagen promoter, we first investigated whether DNA methylation interferes directly with the binding of the transcription factors interacting with the α1(I) collagen regulatory elements described above. FP1 and FP2 were methylated with *Hpa*II methylase, FP4 was methylated with *Hha*I methylase, and the methylated oligonucleotides were used in mobility shift assays (FP3 contains no methylatable CpG; see the sequence in Fig. 2C). The exon I oligonucleotide contains a single central methylatable CpG (Fig. 2C) which was methylated during oligonucleotide synthesis. Figure 6A shows that methylation with *Hpa*II or *Hha*I methylase conferred resistance to the respective restriction enzymes, indicating that the oligonucleotides were completely methylated (for unknown reasons, the unmethylated FP1 and FP2 oligonucleotides regularly showed partial resistance to *Hpa*II). Figure 6B confirms previous observations (51, 52) and shows that the binding of nuclear factors to the FP1 oligonucleotide resulted in distinct complexes, the upper complex representing binding to Sp1 and the lower complex representing binding to NF-1 (51, 52). Identical results were obtained with the FP2 oligonucleotide (data not shown). Figure 6B also shows that both Sp1 and NF-1 were present in nuclear extracts from NIH 3T3 cells, P19 EC cells, and WEHI-3B cells, although in different relative concentrations, and that their binding was not inhibited or was only marginally inhibited by DNA methylation. Similar results were obtained with the FP2 oligonucleotide (data not shown). Figure 6C shows that binding of the factor interacting with the FP4 oligonucleotide was also insensitive to DNA methylation. Similarly, binding of the NF-1-like

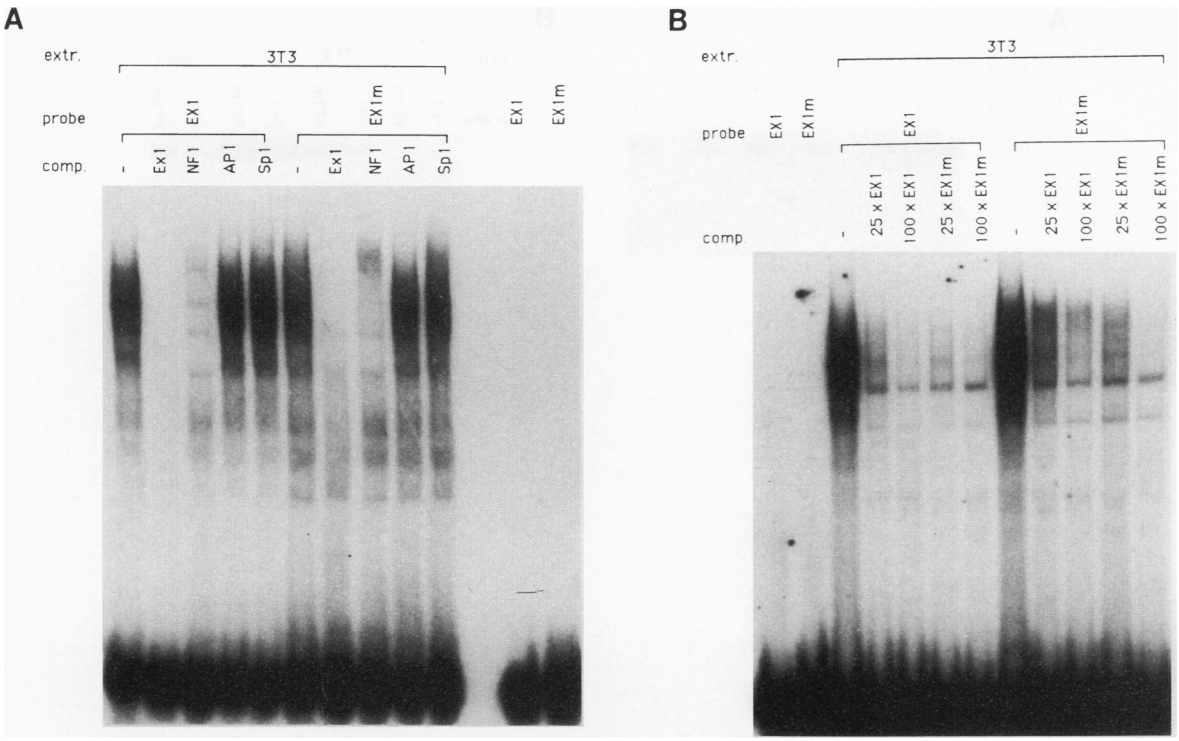

FIG. 5. The regulatory element in the first exon interacts with NF1 in a methylation-independent manner. (A) Unmethylated (EX1) and methylated (EX1m) double-stranded oligonucleotides containing the DNase I-protected region shown in Fig. 2 and 4 were synthesized and radiolabeled. The probes were incubated with no addition or with nuclear extracts (extr.) from NIH 3T3 fibroblasts in the absence or presence of the indicated cold competitor (comp.) oligonucleotides. Complexes were analyzed on 5% nondenaturing acrylamide gels. (B) Unmethylated and methylated EX1 oligonucleotides were incubated without or with NIH 3T3 nuclear extract (extr.) in the absence or presence of the indicated amounts of unmethylated or methylated unlabeled competitor (comp.) oligonucleotides. The complexes were analyzed on 5% nondenaturing acrylamide gels.

factor interacting with the exon oligonucleotide was insensitive to DNA methylation (Fig. 5).

We also compared the relative affinities of the transcription factors for binding to unmethylated and methylated DNA by cross-competition. Figure 5B shows that binding of the exon 1 binding factor to the unmethylated and methylated probes was inhibited equally well by unmethylated and methylated unlabeled oligonucleotides, respectively. Similar results were obtained with the FP1 oligonucleotide (data not shown), confirming that Sp1 and NF-1 bind equally well to their unmethylated and methylated binding sites. The experiments described in the next paragraph show that Sp1 and NF-1 not only bind but also transactivate unmethylated and methylated templates equally well. Thus, none of the transcription factors interacting with the regulatory elements in the α1(I) proximal promoter and first exon are directly inhibited by DNA methylation, suggesting that DNA methylation represses α1(I) promoter activity by an indirect mechanism.

DNA methylation inhibits α1(I) promoter activity in murine EC cells and in *Drosophila* cells. Possible indirect mechanisms of methylation-mediated transcriptional repression are the assembly of methylated templates into inactive chromatin structure or repression by binding of methyl-C-binding proteins. To distinguish between these possibilities, we performed transfection experiments using murine EC cells and *Drosophila* SL2 cells for the following reasons: it has been reported that transcriptional repression of several eukaryotic promoters is mediated by methyl-C-binding protein 1 (MeCP-1) and that murine EC cells contain no or very low concentrations of MeCP-1 (47). Similarly, *Drosophila* DNA is not methylated (54), and cells from such organisms presumably do not contain methyl-C-binding proteins. Therefore, if the methylation-induced repression of the α1(I) collagen promoter is mediated by MeCP-1 or another methyl-C-binding protein, one would expect methylation to have no effect on α1(I) promoter activity in either EC cells or SL2 cells. On the other hand, if an assembly of the methylated template into an inactive chromatin structure independent of methyl-C-binding proteins is responsible for the methylation-induced transcriptional repression, one would expect methylation to inhibit the transcriptional activity of the α1(I) promoter in these cells. To test this, we first transfected unmethylated and methylated α1(I) collagen promoter reporter gene constructs into P19 and F9 EC cells. The results of these experiments showed that α1(I) promoter activity was inhibited by DNA methylation in both EC cell lines, although to a lesser extent than in NIH 3T3 cells. In F9 EC cells (Fig. 1) and P19 EC cells (data not shown), methylation with *Hha*I methylase reduced α1(I) promoter activity only marginally, and methylation with *Hpa*II or *Sss*I methylase inhibited the α1(I) promoter five- and eightfold, respectively.

Because these results did not rule out that the inhibitory effect of methylation was mediated by the low amounts of MeCP-1 that may be present in EC cells (47), we also transfected the unmethylated and methylated reporter gene constructs into *Drosophila* SL2 cells. Because these cells do not contain the mammalian transcription factors Sp1 and NF-1, expression vectors producing these factors were cotransfected

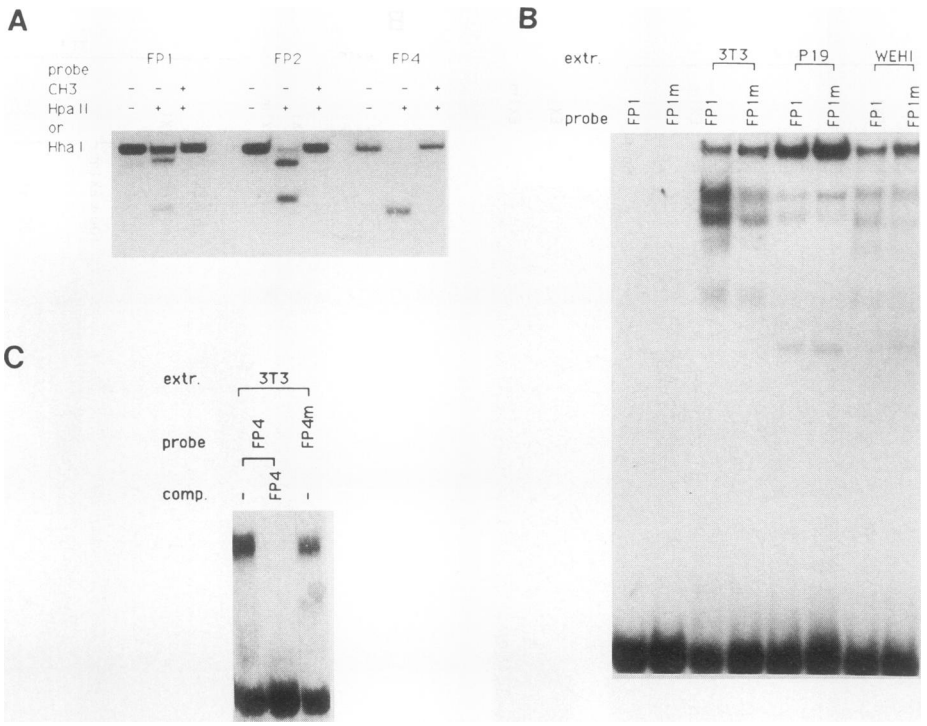

FIG. 6. DNA binding of the α1(I) promoter binding factors is not inhibited by DNA methylation. (A) Double-stranded radiolabeled oligonucleotides containing FP1, FP2, or FP4 were methylated with *Hpa*II (FP1 and FP2) or *Hha*I (FP4) methylase, and completeness of methylation was indicated by resistance to digestion with the appropriate restriction enzyme. For unknown reasons, digestion of the unmethylated FP1 and FP2 oligonucleotides was regularly incomplete. (B) Unmethylated or methylated FP1 oligonucleotides were used for mobility shift assays with NIH 3T3, P19, or WEHI-3B nuclear extracts as indicated. (C) Unmethylated or methylated FP4 oligonucleotides were used for mobility shift assays with NIH 3T3 nuclear extracts in the presence or absence of cold competitor DNA as indicated. All complexes were analyzed on 5% nondenaturing acrylamide gels. Extr., extract; comp., competitor.

as previously described (51). As a control, plasmid pBLCAT19, which contains a promoter than can be transactivated by the transcription factors Sp1 and AP-2 (66), was transfected in parallel experiments together with Sp1 and AP-2 expression vectors. Binding of the transcription factor AP-2 to its target sequence has previously been shown to be directly inhibited by DNA methylation (19). The results of these experiments (Fig. 7) show that methylation did inhibit the α1(I) promoter in SL2 cells. The basal activity of the α1(I) promoter (i.e., in the absence of cotransfected Sp1 expression vector) was inhibited about 5-fold by *Sss*I methylase (Fig. 7A; note that the basal promoter activity was ~50-fold lower than the activity in the presence of a transactivator in the experiment shown in Fig. 7A and B). In the presence of cotransfected Sp1 expression vector, methylation with *Hpa*II methylase inhibited the α1(I) promoter by about 30% and methylation with *Sss*I methylase inhibited it three- to fivefold (Fig. 7B). However, both the unmethylated and methylated Col3luc plasmids were equally well transactivated by Sp1 (sevenfold) and by NF-1 (threefold) (Fig. 7C). Cotransfection with larger amounts of the Sp1 expression plasmid resulted in higher levels of transactivation of both the unmethylated and methylated α1(I) reporter gene construct (50-fold [Fig. 7B]). Similarly, Sp1 stimulated both unmethylated and methylated plasmid pBLCAT19 (70- and 120-fold, respectively [Fig. 7D]). In contrast, AP-2 was able to stimulate only unmethylated (10-fold) but not methylated (0.8-fold) pBLCAT19 promoter (Fig. 7D). These results confirm that DNA methylation does not directly interfere with DNA binding of or transcriptional activation by the transcription factors Sp1 and NF-1, as directly shown in Fig. 6, whereas it does interfere with transcriptional activation by AP-2, as reported earlier (19). These results further indicate that methylation inhibits the transcriptional activity of the α1(I) promoter by an indirect mechanism.

Activity of the methylated promoter can be restored by an excess of unspecific, methylated DNA. It has been reported that the methylation-induced repression of transcription by histone H1 (44) or methyl-C-binding proteins (8, 42) can be reversed by cotransfection with methylated, promoterless DNA. To test this, we performed rescue cotransfection experiments in which methylated Col3luc was cotransfected with unmethylated and methylated *M. luteus* DNA. Figure 8 shows that cotransfection of methylated competitor DNA completely restored the activity of the *Hha*I-methylated Col3luc and partially restored the activity of the *Hpa*II-methylated Col3luc. However, we were not able to rescue promoter activity when the constructs were methylated to a higher density with *Sss*I methylase (data not shown). A similar ability to rescue transcriptional activity of only a lightly but not more heavily methylated promoter by cotransfection with unspecific methylated competitor DNA has been described by others (8), although they were able to rescue the activity of heavily methylated promoters in in vitro transcription experiments.

DISCUSSION

The molecular mechanisms by which DNA methylation interferes with gene expression are only partly understood. It is

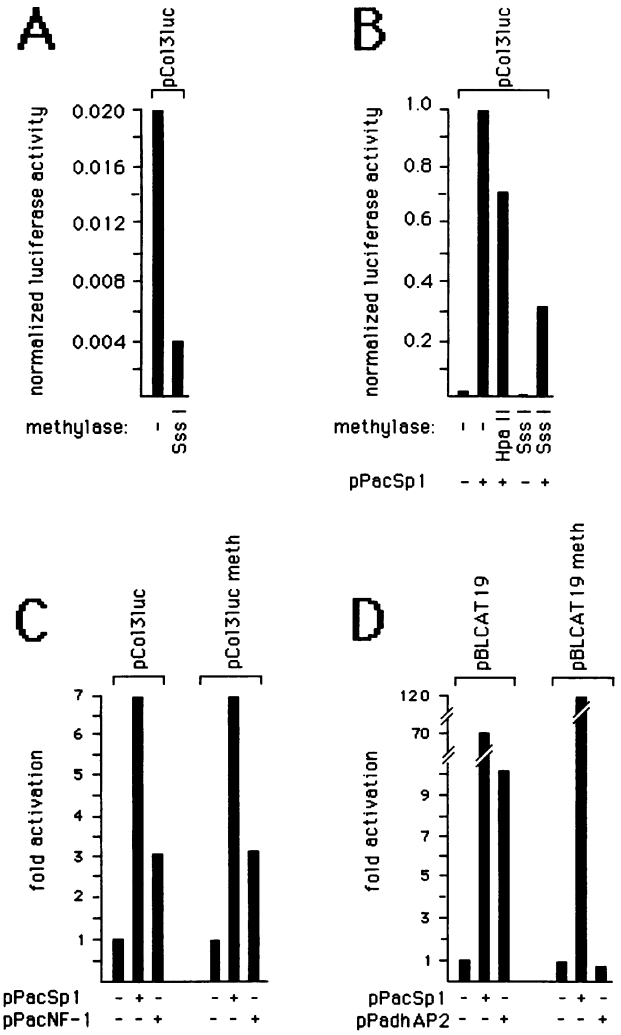

FIG. 7. DNA methylation inhibits α1(I) promoter activity in *Drosophila* SL2 cells. (A) Plasmid pCol3luc was methylated or mock methylated with *Sss*I methylase, and 2 μg was transfected into SL2 cells in the absence of the Sp1 expression plasmid. Note that the data shown are the same as those in columns 1 and 5 of panel B on an extended scale and that the basal promoter activity was ~50-fold lower than in the presence of the Sp1 transactivator. (B) Plasmid pCol3luc was methylated or mock methylated with *Sss*I or *Hpa*II methylase, and 2 μg was transfected without or with 1 μg of Sp1 expression plasmid into SL2 cells. (C) Plasmid pCol3luc was methylated or mock methylated with *Sss*I methylase, and 2 μg was transfected into SL2 cells in the absence or presence of Sp1 (0.1 μg) or NF-1 (4 μg) expression plasmid as indicated. (D) Plasmid pBLCAT19 was methylated or mock methylated with *Sss*I methylase, and 2 μg was transfected without or with the indicated Sp1 (0.1 μg) or AP-2 (2.5 μg) expression vector into SL2 cells. pRSVβ-gal was included in all transfections to normalize for transfection efficiency. Luciferase or CAT activities were determined and normalized for transfection efficiencies. In panels C and D, activities are shown as fold activation of the unmethylated and methylated constructs by the Sp 1, NF-1, or AP-2 expression plasmids.

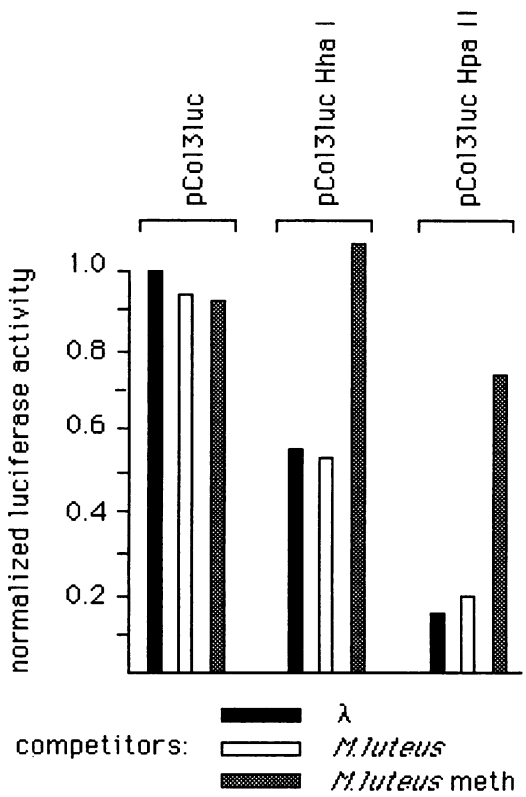

FIG. 8. Methylation-induced transcriptional repression can be partially reversed by unspecific, methylated competitor DNA. Plasmid pCol3luc was mock methylated or methylated with *Hha*I or *Hpa*II methylase, and 1 μg was cotransfected into NIH 3T3 cells with pRSVβ-gal and 15 μg of λ DNA, *M. luteus* DNA, or *Sss*I-methylated (meth) *M. luteus* DNA as indicated. Normalized luciferase activity was determined as described in the legends to Fig. 1 and 7.

generally accepted that DNA methylation alters DNA-protein interactions and can directly interfere with the binding of transcriptional activators or repressors or more indirectly affect the structure of chromatin or facilitate the binding of methyl-C-binding proteins, i.e., proteins that bind in a sequence-independent manner to methylated but not unmethylated DNA. The results of the experiments described in this paper strongly suggest that a direct mechanism is not responsible for the methylation-induced repression of the murine α1(I) collagen promoter. Mobility shift experiments (Fig. 5 and 6) show that none of the factors interacting with the promoter and first exon regulatory elements are directly inhibited from binding to their recognition sequences when the sequences are methylated. In addition, transcriptional activation of the α1(I) promoter by its main transactivators Sp1 or NF-1 is not inhibited by DNA methylation (Fig. 7). We also found that the methylation-induced repression of the α1(I) promoter can be partially reversed by cotransfection with unspecific, methylated competitor DNA (Fig. 8), which has previously been shown to reverse the methylation-induced repression by histone H1 (44) and methyl-C-binding proteins (8, 42). Thus, DNA methylation appears to inhibit the α1(I) promoter through an indirect mechanism.

To elucidate which of the indirect mechanisms—either assembly into inactive chromatin or inhibition by methyl-C-binding proteins—represses the transcriptional activity of the methylated α1(I) promoter, we transfected methylated α1(I) reporter gene constructs into EC cells, which have been reported to contain very little or no MeCP-1 (47), and into *Drosophila* SL2 cells, which also presumably contain no methyl-C-binding proteins. In both cell types, α1(I) promoter activity was inhibited by DNA methylation (Fig. 1 and 7), although to a much lesser extent than in NIH 3T3 fibroblasts.

This indicates that methylation can repress the α1(I) promoter by a mechanism independent of methyl-C-binding proteins, presumably by promoting assembly of methylated DNA into an inactive chromatin structure. These results also suggest that in NIH 3T3 cells (and other differentiated vertebrate cells), chromatin structure and methyl-C-binding proteins may cooperatively mediate the inhibitory effect of DNA methylation (see below). Several lines of evidence support the assumption that chromatin structure is involved in mediating the inhibitory effect of DNA methylation on transcriptional activity: (i) transfected methylated DNA is more resistant to endonucleases than unmethylated DNA (39); (ii) chromatin formation is required to block transcription of microinjected methylated DNA (14); (iii) 5-methyl-cytosine is localized primarily in inactive nucleosomes containing histone H1 (2); and histone H1 shows stronger binding to and transcriptional inhibition of methylated templates than it does with regard to unmethylated templates (44).

As mentioned above, the methylation-induced repression of the α1(I) promoter was always 5- to 10-fold lower in EC and SL2 cells than in NIH 3T3 cells. This suggests that in differentiated vertebrate cells, both chromatin and methyl-C-binding proteins may play a role in methylation-induced transcriptional repression, although it is not known what the interrelationship between these two mechanisms in vivo is. Do methyl-C-binding proteins promote the assembly of methylated DNA into condensed, inactive chromatin, or is the assembly of methylated DNA into condensed chromatin a prerequisite for binding of methyl-C-binding proteins? Our results are in support of although no proof for the latter interpretation. In the absence of methyl-C-binding protein (such as in *Drosophila* cells), the DNA may be preferentially packaged into inactive chromatin, presumably involving histone H1. This results in an incomplete repression of transcription and allows a fraction of the methylated DNA still to be transactivated by methylation-insensitive transcription factors such as Sp1 and NF-1. This result is in agreement with a recent finding (43) which suggests that once a transcriptional preinitiation complex has assembled, several rounds of transcription with normal reinitiations can occur, independently of the methylation status of the promoter. In vertebrate cells, the binding of methyl-C-binding proteins may then result in complete transcriptional repression by further condensing the chromatin and/or preventing the transcriptional machinery from binding. Such a mechanism may also explain an interesting feature of a well-characterized methyl-C-binding protein, MeCP-1. This protein has been reported to require 10 to 15 methyl-CpGs in a DNA fragment of ~150 bp in order to bind DNA (47). However, vertebrate DNA is five- to sixfold depleted of CpG dinucleotides and has therefore only about 2 CpGs per 150 bp. The CpG density required for MeCBP-1 binding is found only in CpG-rich islands, which are usually unmethylated (5). The condensation of methylated DNA into inactive chromatin may lead to an increased local methyl-CpG density and may thus be a prerequisite for MeCBP-1 binding.

An indirect mechanism of methylation-induced transcriptional inhibition of promoter activity would not require a promoter itself to be methylated to be repressed. Several observations are consistent with this assumption. For example, an analysis of the effect of DNA methylation on expression of the human γ-globin gene (50) has shown that the extent rather than the site of methylation is important for transcriptional repression. Similarly, the transcriptional activity of the simian virus 40 early promoter appears to depend on the vector or reporter gene used (12, 26, 63). A recent study indicates that an inactive chromatin conformation can spread from a focus of methylation (38). Finally, we have previously shown that the area downstream of the α1(I) promoter and not the promoter itself is differentially methylated in collagen-producing and nonproducing cells and tissues (57). Thus, the simplest model to interpret our data on how DNA methylation affects α1(I) promoter activity is to assume that DNA methylation in the vicinity of the α1(I) promoter induces local chromatin condensation which facilitates binding of methyl-C-binding protein, rendering the promoter inaccessible to the ubiquitous transcription factors and resulting in transcriptional repression.

However, we cannot rule out that in situ both direct and indirect mechanisms contribute to the methylation-induced repression of the α1(I) promoter. It has been suggested that a combination of three different parameters determines the effect of DNA methylation on gene expression: the strength of a promoter, the CpG density, and the location of methylated sites relative to the promoter (9, 43, 50). The mobility shift and transactivation experiments (Fig. 5, 6, and 7) strongly suggest that methylation does not weaken the α1(I) promoter by directly preventing binding of a factor(s) contributing to promoter strength. Similarly, the findings that Sp1 and NF-1 can transactivate the methylated and unmethylated constructs equally well (Fig. 7) and that the activity of the *Hha*I-methylated construct can be completely restored by unspecific, methylated competitor DNA (Fig. 8) indicate that methylation does not per se weaken the α1(I) promoter. On the other hand, the CpG density and location may be of more critical importance. The promoter region of the α1(I) gene is relatively although not dramatically depleted of CpG dinucleotides compared with GpC dinucleotides (Fig. 2), and methylation of all CpGs with *Sss*I methylase almost completely abolishes promoter activity (Fig. 1, 7, and 8). It has been shown previously that sparsely methylated promoters can be inhibited by MeCBP-1 and that this effect can be overridden by a strong enhancer (9). A likely explanation for the strong effect of *Sss*I methylation in our experiments is that the α1(I) promoter construct used (Col3luc) does not have a discrete enhancer. The inhibitory effect of methylating the single *Hha*I site and, to a greater extent, the *Hpa*I sites (Fig. 1, 7, and 8) may reflect the critical positionings of these sites in the promoter; the *Hha*I site is located in FP4, and the two *Hpa*II sites are located in FP1 and FP2, respectively (Fig. 2). Previous studies have clearly demonstrated the importance of the positioning of the methylated cytosines near the site of transcriptional preinitiation complex formation (43). Additional experiments are necessary to further address these questions.

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