Biochem. J. (1992) 283, 699–703 (Printed in Great Britain)

DNA methylation inhibits transcription of procollagen α2(I) promoters

Denis K. GUENETTE, Jeffrey D. RITZENTHALER, Joe FOLEY, John D. JACKSON and Barbara D. SMITH*
Department of Biochemistry at Boston University School of Medicine and the Boston Veterans Administration Medical Center, Boston, MA 02118, U.S.A.

Our previous studies have demonstrated that a 2-[*N*-(acetoxyacetyl)amino]fluorene-transformed rat epithelial-like cell line, W8, contains a transcriptionally inactive α2(I) gene with a hypermethylated promoter/first-exon region. We have cloned the rat promoter/first-exon region (−211 to +207) from W8 cells and their parent cell line, K16, which expresses α2(I) collagen. There were no sequence differences between the clones from the two cell lines, indicating that a mutation was not responsible for transcriptional inhibition. The α2(I) rat promoters were cloned upstream of the chloramphenicol acetyltransferase gene. Both constructs were equally active in both cell lines, suggesting that *trans*-activating factors for α2(I) transcription are present in W8 cells. Finally, methylation of plasmids at all CpG sites with *Sss*I methylase completely inhibited transcription using α2(I) promoters, but methylation did not inhibit simian-virus-40 promoter-driven transcription. Certain methylation sites partially inhibit promoter activity. An *Hha*I methylation site inhibited transcriptional activity of the α2(I) promoter 8-fold, whereas methylation at the *Hpa*II site in the rat α2(I) promoter did not decrease transcriptional activity. This provides further evidence that methylation at specific sites in the collagen α2(I) promoter is responsible for the inactivation of transcription in W8 cells.

INTRODUCTION

Collagen type I, the most abundant collagen molecule within the collagen family, normally consists of a heterotrimer with two α1(I) chains and one α2(I) chain. This form of collagen represents the major fibrillar component of connective tissue [1]. Usually type I collagen chains are co-ordinately expressed. However, occasionally in tumours [2,3] or in cell cultures [4], a homotrimer of α1(I) chains occurs, referred to as α1(I) trimer.

Usually cells transformed in culture into tumorigenic cell lines produce less collagen [5–9]. Viral transformation of fibroblasts decreases mRNA levels for both collagen type I α-chains [6,7] by decreasing collagen transcription [7,9]. Chemical transformation of cells also decreases collagen synthesis [8,10]. In the case of 4-nitroquinoline-1-oxide-transformed Syrian-hamster fibroblasts, the α1(I) chain is not expressed and an altered α2(I) chain is secreted [10]. Those authors [10] suggested that mutations have occurred, inhibiting regulation of the α1(I) chain and allowing secretion of an altered α2(I) chain.

We have been investigating a 2-[*N*-(acetoxyacetyl)amino]-fluorene (AAF)-transformed rat liver epithelial-like cell line, W8, and its parent cell line, K16 [4,11–13]. Previous studies [4] indicated that K16 cells, like other rat liver epithelial-like cells in long-term culture [14], produce primarily type I collagen. However, the chemically transformed W8 cells produce α1(I) trimer.

In order to identify the mechanism for the lack of α2(I) gene expression, we examined α2(I) mRNA levels and gene structure in W8 cells. W8 cells do not transcribe mRNA for α2(I), as judged by translation assay [11], Northern-blot analysis [11], S1-nuclease assays [15], and 'nuclear run-off assays' [12]. We have also demonstrated [12] that the W8 α2(I) gene is present with no large insertions or deletions. However, the 5′ region of the W8 α2(I) gene is hypermethylated, whereas the similar region in K16 cells is not methylated. Transcription of the α2(I) mRNA is induced using azacytidine, an inhibitor of DNA methylation, indicating that DNA methylation altered α2(I) transcription. Methylation of DNA correlates with loss of transcriptional activity [16,17], and methylation of critical regions of promoters can inhibit transcription of certain genes [18].

Here we demonstrate that transcriptional activity of α2(I) collagen promoters [−345 or −218 to +58 collagen α2(I) genes] cloned upstream of a reporter gene, *CAT*, coding for chloramphenicol acetyltransferase (CAT), can be inhibited by methylation of the plasmids. We have cloned and sequenced 200 bases of promoter and the whole first exon of rat α2(I) collagen gene from W8 and K16 cells. There were no differences between the K16 and W8 α2(I) promoter/first-exon sequences. In addition, both rat promoters and the human α2(I) promoter can drive transcription of a reporter gene, *CAT*, after transfection into W8 cells, similar to our previously described results with the mouse α2(I) promoter [13]. These findings suggest that DNA methylation, not mutations or alterations in *trans*-acting factors, inhibits transcription in the W8 cells and that methylation plays a role in abrogation of α2(I) gene expression.

MATERIALS AND METHODS

Cell culture

Rat liver epithelial cells (K16) and chemically transformed cells (W8) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin G/streptomycin sulphate, 1% sodium pyruvate and 1% L-glutamine as described previously [4,11–13].

Cloning of promoter/first exon into pBluescript and expression vectors

Genomic DNA was extracted from cell cultures by standard methods [19], by using proteinase K (0.1 mg/ml) in 0.5% SDS/25 mM-EDTA/100 mM-NaCl/10 mM-Tris/HCl, pH 8. DNA from K16 and W8 cells was further purified by phenol/chloroform/3-methylbutan-1-ol extraction and precipitated with ethanol (2 vol.) with ammonium acetate (0.5 vol. of 7.5 M-

Abbreviations used: AAF, 2-[*N*-(acetoxyacetyl)amino]fluorene; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; TE, 10 mM-Tris/HCl/1 mM-EDTA, pH 8.0; pBS, pBluescript.

* To whom correspondence should be sent.

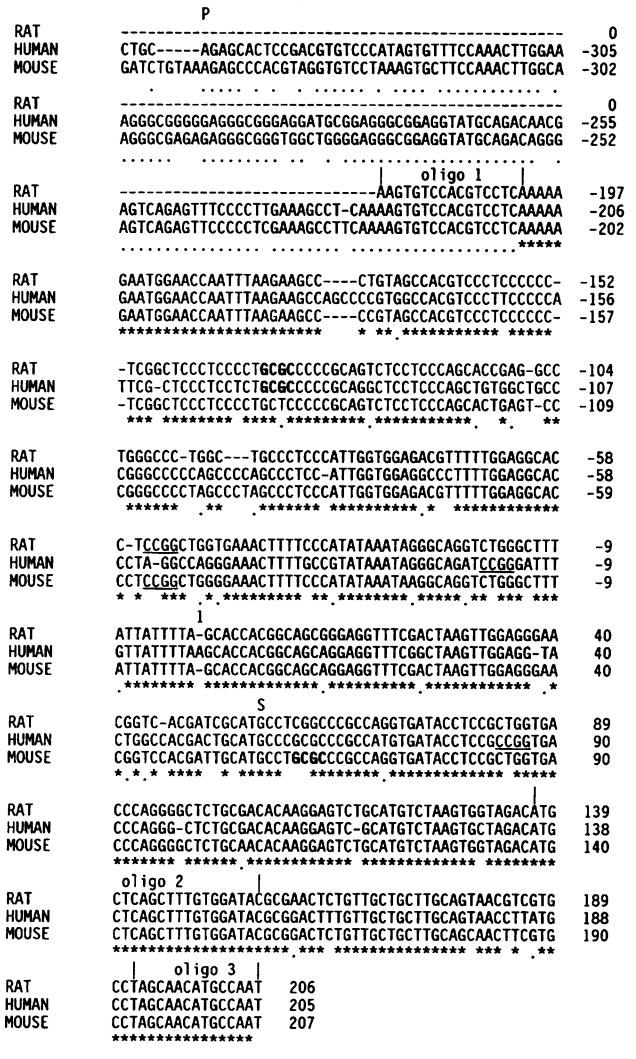

Fig. 1. Sequence comparison of α2(I) promoter/first exon from rat, human and mouse

Key to symbols: *, perfectly conserved position; ·, well-conserved position; P, *Pst*I site, CCGG, *Hpa*II sites; **GCGC**, *Hha*I sites; S, *Sph*I site; 1, transcription start site; |oligo *n*|, sequences used for PCR.

ammonium acetate). After centrifugation the pellet was re-suspended in 10 mM-Tris/HCl/1 mM-EDTA, pH 8 (TE). Each sample was electrophoresed on a 1%-agarose gel to detect degradation and/or RNA contamination.

DNA (1 μg) from each cell line was subjected to amplification by PCR using Perkin–Elmer Cetus GeneAmp DNA Amplification Kit (Perkin–Elmer Cetus Corporation, Norwalk, CT, U.S.A.) according to the manufacturer's recommendations. The oligonucleotide primers to a promoter region (PCR oligo 1 in Fig. 1) conserved between mouse and human α2(I) promoters and the last 15 bases in the first exon (complementary bases to PCR oligo 3 in Fig. 1) were synthesized (Oligo etc., Ridgefield, CT, U.S.A.). The temperatures for PCR were 94 °C denaturation temperature (1 min), 42 °C annealing temperature (2 min), and 72 °C extension temperature (2 min) for 30 cycles. The reaction mixtures were examined by 1%-agarose-gel electrophoresis. Southern-blotted and hybridized to an internal 3′-end-labelled primer (internal oligo 3 in Fig. 1). The 424 bp amplified product was separated from excess oligonucleotides by means of a Centricon 100 apparatus (Amicon, Danvers, MA, U.S.A.) or by elution from an agarose gel, cloned into *Sma*I-digested pBluescript (pBS) plasmid (Stratagene, La Jolla, CA, U.S.A.), and grown in DH5α *Escherichia coli* cells (BRL, Bethesda, MD, U.S.A.). Colourless colonies on MacConkey plates were grown overnight and the plasmid was isolated using a boiling plasmid preparation method [17]. Several different preparations with inserts were sequenced by the Sanger dideoxy sequencing method using Sequenase enzyme (USB, Cleveland, OH, U.S.A.) primed with universal and reverse primers.

After sequencing, clones in the proper orientation were digested with *Pst*I/*Sph*I and subcloned upstream of the *CAT* gene. The plasmid containing the human α2(I) promoter (pMS-3.5) [20] (kindly provided by Dr. F. Ramirez, Mount Sinai School of Medicine, New York, NY, U.S.A.) was restriction-enzyme-digested with *Pst*I and *Sph*I to remove the human α2(I) promoter. The K16 and W8 rat promoters from −218 to +52 were cloned into these CAT constructs, which are p8-CAT derivatives of pEMBL plasmid [21] (plasmid with W8 promoter is pJAR-W220; plasmid with K16 promoter is pJAR-K220). The pMS-3.5 plasmid was also restriction-digested with *Pst*I alone and re-ligated, forming a *CAT* construct, pJAR-H340, containing α2(I) sequences from −345 to +58. This vector was used in these experiments and compared with the rat constructs.

Constructs (pJAR-W220, pJAR-K220, pJAR-H340, and SV2CAT) were methylated by *Sss*I methylase (New England Biolabs, Beverly, MA, U.S.A.), *Hpa*II methylase or *Hha*I methylase by incubating plasmids with enzymes (1 unit/μg of DNA) for 16 h at 37 °C in 200 μl of incubation buffer (*Sss*I incubation buffer was 50 mM-NaCl/10 mM-EDTA/1 mM-dithiothreitol/160 μM-*S*-adenosylmethionine/10 mM-Tris/HCl, pH 8. The *Hpa*II and *Hha*I incubation buffer was 10 mM-EDTA/5 mM-2-mercaptoethanol/80 μM-*S*-adenosylmethionine/50 mM-Tris/HCl, pH 7.5) according to manufacturer's recommendations. The methylation reaction was terminated by incubating the samples at 65 °C for 20 min. Plasmids were precipitated at −70 °C in 2 vol. of ethanol in a final concentration of either 0.3 M-sodium acetate or 2 M-ammonium acetate. The samples were centrifuged, washed with 70% ethanol and redissolved in TE. Before transfection, the methylation reaction was confirmed by digesting 1 μg of plasmid with either *Hpa*II or *Hha*I restriction enzymes and separating the products electrophoretically on 1%-agarose gel. Control plasmids were incubated without methylating enzymes, precipitated and compared with methylated plasmids in transfection studies.

Transient transfections

Confluent W8 and K16 cells were trypsin-treated with 1 ml of trypsin (0.5 g/l)/EDTA (0.2 g/l), plated at a density of (5–8) × 10^5 cells/100 mm^2 tissue-culture dish with 10 ml of media containing 10% serum and incubated in 8% CO_2 at 37 °C. After 24 h the plasmid DNA was transfected into cells by calcium phosphate precipitation [22]. CAT plasmids (10 μg) were co-transfected with β-galactosidase [23] (pRSV-β-gal) (5 μg) to normalize for transfection efficiency. Cells were glycerol-shocked with 15% glycerol for 45 s [22] 16 h after the addition of plasmids. Cells were allowed to grow for 48 h before harvest.

CAT assays

Cells were harvested in 1 ml of 10 mM-EDTA/1 M-NaCl/100 mM-Tris/HCl, pH 8.0. Cell extracts were centrifuged for 3 min (at 1500 *g*), resuspended in 100 μl of 250 mM-Tris/HCl, pH 7.8, and sonicated for 15–20 s with a Branson model 450 sonifier. Total protein was measured by Bradford method using 1 and 2 μl aliquots of cell extracts and Commassie Blue G-250 reagent [24].

CAT activity was measured as described by Gorman *et al.*

[22]. Radiolabelled chloramphenicol (0.25 μCi of D-*threo*-[*dichloroacetyl*-1-^{14}C]chloramphenicol) and 4 mM-acetyl-CoA were added to samples containing 100 μg of protein or CAT enzyme in 150 μl of 0.25 M-Tris/HCl, pH 7.8. Samples were incubated at 37 °C for 4–6 h, which was within the 'linear' range. After ethyl acetate extraction, the chloramphenicol and the acetylated products were separated by t.l.c. for 1.25 h in chloroform/methanol (19:1, v/v). The thin-layer plates were exposed to X-ray film for autoradiography. Acetylated and non-acetylated radioactive areas were removed from the thin-layer plates, placed into scintillation-counting fluid and quantified on a Beckman scintillation counter.

Experimental transfection efficiency for experiments was determined by co-transfection with Simian-virus-40 (SV40)-driven β-galactosidase gene (SVβgal) (kindly provided by Dr. N. Rosenthal, Boston University School of Medicine, Boston, MA, U.S.A.) [23]. Sonicated-cell extract (30 μl) was incubated at room temperature in a solution containing 1.0 mM-$MgCl_2$, 15 mM-β-mercaptoethanol, 3.0 mM-2-nitrophenyl-β-D-galactopyranoside, and 70 mM-$NaHPO_4/Na_2HPO_4$, pH 7.4. The reaction was terminated after 16 h with 500 μl of 1 M-Na_2CO_3 and the A_{410} was measured.

RESULTS

Our previous data suggested that methylation inhibited the transcription of the α2(I) collagen. However, DNA methylation could have occurred after transcriptional inactivation by a mutation within the promoter or first exon. Therefore it was necessary to clone and sequence the α2(I) promoter/first-exon region from the W8 cells and the parent cell line, K16. Since this region of the rat gene had not been previously cloned and sequenced, primers for PCR were chosen that are fully conserved between human and mouse (see Fig. 1 for sequences). PCR was performed on both K16 and W8 DNA in order to amplify a portion of the promoter and the whole first exon. There was a major amplified product at the expected size (approx. 400 bp) on a 2%-agarose gel (Fig. 2*a*). The W8 sample contained additional bands; therefore the DNA was transferred to nitrocellulose and hybridized to an internal well-conserved oligonucleotide found within the first exon. A single amplified radiolabelled product was detected for each genomic DNA sample (Fig. 2*b*). The PCR products were extracted from agarose gels and cloned into *Sma*I digested pBS plasmids as described in the Materials and methods section.

Both K16 and W8 clones had the same sequence throughout, indicating that there were no point mutations or frameshifts in the W8 α2(I) gene in this region. Several different clones were sequenced in both directions to be certain that no mutations could be demonstrated. Fig. 1 shows the sequence for the rat promoter/first exon in comparison with mouse and human species. As expected, the rat sequence is more closely related to the mouse than the human sequence.

In order to test promoter activity, presence of *trans*-acting factors in both cell lines, and confirm by function that there are no sequence differences, both promoters (−218 to +58) were cloned upstream of *CAT*. As demonstrated in Fig. 3, both promoters were equally active in the W8 and K16 cells. This is confirmatory evidence that a mutation in this portion of the gene has not inactivated the promoter and that the *trans*-acting factors necessary for α2(I)-gene expression are present in the W8 cells.

Next, to test whether methylation will inhibit promoter activity in these constructs, plasmids were incubated in buffer with and without methylating enzymes, *Sss*I methylase, *Hpa*II methylase and *Hha*I methylase. Completeness of methylation was tested by treating an aliquot of the methylated and mock-methylated plasmids with the appropriate restriction enzymes. Fig. 4 displays typical restriction digestions of aliquots from samples used for transient transfection experiments. The methylated constructs are protected from restriction digestion with either *Hpa*II or *Hha*I (Fig. 4). Only completely methylated constructs protected from restriction digestion were transfected into W8 cells.

The α1(I) promoter activity in methylated plasmids was tested by transfection assays. As Fig. 5 shows, the *Sss*I methylase, an enzyme that methylates all CpG sites, did not alter CAT activity in the SV40 plasmids. This control indicates that methylation of the plasmid sequences, including the CAT gene and SV40 promoter, does not interfere with *CAT* transcription. On the other hand, methylation completely inactivated the human and rat α2(I) promoter activity. This indicates that the α2(I) promoter is sensitive to methylation, since the only difference between plasmids was the promoters. Each duplicate assay represented a

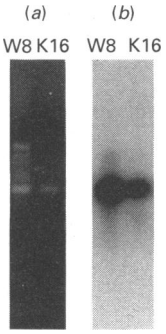

Fig. 2. PCR-amplified products separated by 1.2%-agarose-gel electrophoresis

(*a*) Ethidium bromide-stained gel; (*b*) Southern blot of the same gel hybridized to end-labelled oligo 2 in Fig. 1.

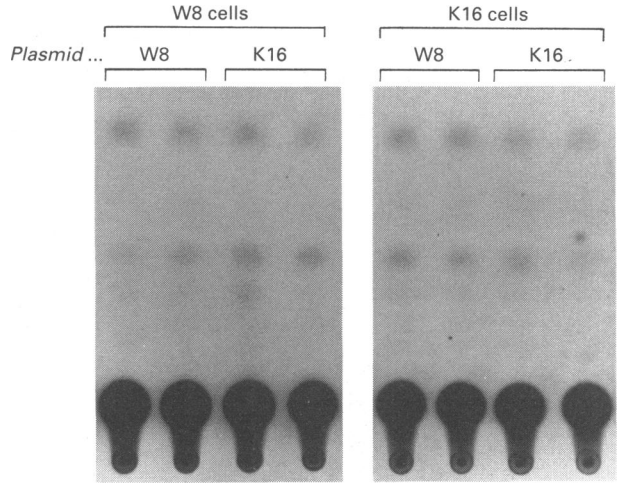

Fig. 3. CAT assay of α2(I) rat promoter from W8 and K16 DNA transfected in W8 and K16 cells

Transfections and CAT assays were performed as described in the Materials and methods section. Each duplicate assay represents a CAT assay on a separate flask. T.l.c. of CAT assay mixtures separated acetylated chloramphenicol from unacetylated chloramphenicol after 4 h incubations. Products on the thin-layer chromatographs were revealed by fluorography and were quantified by removal of radioactive areas and counting them in a scintillation counter. The acetylated chloramphenicol (c.p.m./μg of protein) by all promoters in W8 cells was 648 ± 8.33 (S.E.M.) and in K16 cells it was 892 ± 125 (S.E.M.). The efficiency of transfections as measured by β-Gal assay was the same for all [0.06495 ± 0.003 (S.E.M.) *A*].

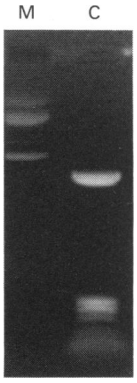

Fig. 4. *Hha*I-digested constructs protected by methylation (M) or unmethylated controls (C) on 1%-agarose gel

Duplicate aliquots were incubated in the buffer described in the Materials and methods section with (M) and without (C) *Hha*I methylase. After incubation and ethanol precipitation, 1 μg was digested with *Hha*I restriction enzyme and the products were separated on a 1%-agarose gel.

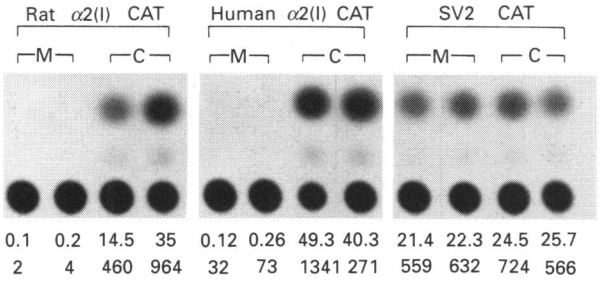

Fig. 5. CAT assay of *Sss*I methylated (M) and unmethylated (C) plasmids transfected in W8 cells

Three plasmids, human α2(I) CAT (pJAR H340), rat α2(I) CAT (pJAR W220) and SV2 CAT, were methylated by *Sss*I enzyme or incubated without enzyme as described in the Materials and methods section. After indication that methylation was complete (Fig. 4), the plasmids were transfected in duplicate flasks of W8 cells. CAT enzyme assays were performed as detailed in the Materials and methods section. The acetylation in each lane is shown below each lane as percentage acetylation [('acetylation' c.p.m./total recovered c.p.m.) × 100] (*a*) and 'acetylated' c.p.m./μg of total protein in the assay (*b*). The average β-Gal activity for all twelve assays in this experiment was 0.105 ± 0.01 (S.D.) *A*.

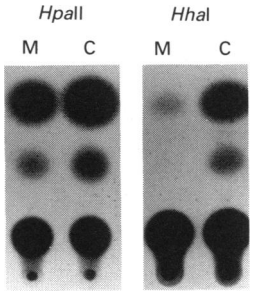

Fig. 6. CAT assay of *Hpa*II and *Hha*I methylated (M) and unmethylated (C) plasmids transfected into W8 cells

Methylated (M) and unmethylated (C) α2 promoter CAT constructs were transfected into W8 cells. The *Hpa*II experiment was performed by methylating the human promoter construct (pJAR-H340) with *Hpa*II methylase. The *Hha*I experiment was performed by methylating the rat W8 promoter construct (pJAR-W220) with *Hha*I methylase. These are representative CAT assay results from three separate experiments.

separately transfected flask performed with 100 μg of protein per assay. The β-Gal plasmid was used as a control for possible variability in transfection. The β-Gal activity, as measured by the absorbance of the product, was 0.10 ± 0.01 (S.D.), indicating that the transfection efficiency was the same for all the transfections.

When individual sites in plasmids were methylated by more specific methylases, only certain sites could partially inactivate transcription. *Hpa*II methylation, which methylates CCGG sequences, caused a 3-fold decrease in the transcription of the human α2(I) promoter (Fig. 6). There is one *Hpa*II site in the rat and human sequences, but they are in different positions (Fig. 1). There was no change in the rat promoter α2(I) activity (results not shown). Therefore not all methylation sites will inactivate transcription.

The *Hha*I site, CGCG, is conserved in human and rat species, but not in mouse, and is located in a region rich in cytosine bases (Fig. 1). Both rat and human *Hha*I methylation decreased CAT activity 8-fold (Fig. 6). As noted by other investigators [18] and with *Sss*I methylation, the activity of the SV2CAT construct was not altered by *Hpa*II or *Hha*I methylase, indicating that methylation of the SV40 promoter or CAT-gene sequences does not effect transcriptional activity.

DISCUSSION

The W8 cell line was prepared by treating K16 cells with the carcinogen AAF, which forms an adduct on guanine [25] and, in bacterial systems, can cause a mutation substitution for guanine or deletion mutations [26]. Therefore, one reason for alterations in α2(I)-gene expression could be a point mutation in transcriptional regulatory regions or a frameshift mutation in the first exon. Our sequence data indicate no mutations between the α2(I) genes from the treated and untreated cells. Obviously a mutation could be present in another regulatory region that has not yet been sequenced. However, most examples of mutations causing a transcriptional inactivation are close to the transcriptional start site or present in, or near, the first exon.

Our earlier data demonstrated that normal α2(I) promoters from mouse [13] function equally well in K16 and W8 cells. Other investigators demonstrated that this mouse construct was sensitive to transactivating factors, decreasing collagen transcription after viral transformation of fibroblasts [27]. However, the full-length (2000 bp) mouse α2(I) promoter *CAT* construct did not have lower activity in W8 cells than K16 cells [13]. Stably transfected W8 cells are able to express CAT with the full-length mouse α2(I) promoter [13]. The present paper demonstrates further that human and rat α2(I) promoters also function within the W8 cells, even though the α2(I) gene is not expressed. A full-length human (3600 bp) α2(I) promoter *CAT* construct (result not shown), as well as the 340-bp shorter human α2(I) construct, is active in W8 cells. These results strongly suggest that W8 cells contain the necessary *trans*-acting factors for transcription, but that *cis*-alterations, such as methylation, in the promoter have inactivated transcription. Although there is always a possibility that the constructs used in our studies do not contain all regulatory elements, we used the available constructs to test whether methylation can inactivate the α2(I) promoter.

Numerous examples indicate that there is an inverse correlation between DNA methylation and the level of gene expression [16,17,18], i.e. active genes are hypomethylated and inactive genes are often hypermethylated. Recent evidence suggests that not all methylation sites are critical for transcription [18]. Certain sites near transcriptional start sites in promoter/first-exon regions have been demonstrated to inactivate transcription.

The relationship between methylation and collagen transcription has been controversial. Early investigations [28,29] demon-

strated that the methylation state of the chicken α2(I) gene did not correlate with alterations in gene expression, and the 5′ region of chicken α2(I) gene was not methylated in cells that did not express collagen. However, we [12] and others [30,31] have observed an increase in collagen-gene DNA methylation with a decrease in collagen expression in certain transformed cell lines. Our previous data demonstrated that W8 cells, which do not express α2(I) collagen mRNA, are hypermethylated in the 5′ region of the α2(I) gene [12] at several *Hpa*II sites. Most important, azacytidine, an inhibitor of methylation, induces transcription of the W8 α2(I) gene [12]. Similar studies have been published indicating that azacytidine can induce transcription of the α1(I) collagen gene in rhabdomyosarcoma cell line [28].

Transformation is known to alter methylation patterns of cells, generally causing an overall hypomethylation of the genome with the concomitant hypermethylation of some regions of the genome [32]. In W8 cells, the α2(I) 5′-region is hypermethylated, in contrast with other portions of the α2(I) gene, the α1(I) gene and the tubulin gene, which remain similar or are less methylated [12].

The present paper demonstrates that the α2(I) promoter is sensitive to DNA methylation. Complete methylation of all CpG sequences inactivated the α2(I) promoters from human and rat sources. We have also explored the alterations in transcription by methylation at different sites in the α2(I) promoter. We have demonstrated that the *Hha*I site, a conserved site in human and rat α2(I) promoters, inhibits transcription 8-fold. On the other hand, methylation of the *Hpa*II site, which is not in the same region in human and rat α2(I) promoters, reduces transcription in human α2(I) promoter, but not in the rat promoter. Therefore, not every site will inactivate transcriptional activity. Others using similar techniques have shown that the human α1(I) promoter is also sensitive to methylation [28]. Not all promoters are sensitive to methylation; for example, the SV40 promoter used in these experiments was not inactivated by *Sss*I methylase. DNA methylation could inactivate transcription by interfering with chromatin structure or by altering binding of transcriptional regulatory proteins [16–18].

This work was supported by a grant R01-CA23540 awarded by the National Cancer Institute, National Institutes of Health, and supported in part by grant no. R01-HL41373 from the National Heart, Lung and Blood Institute, National Institutes of Health, and Medical Research Services of the Veterans Administration.

REFERENCES

1. Mayne, R. & Burgeson, R. E. (eds.) (1987) Structure and Function of Collagen Types, Academic Press, London and New York
2. Moro, L. & Smith, B. D. (1977) Arch. Biochem. Biophys. **182**, 33–41
3. Minafra, S., Pucci-Minafra, I., Tomasino, R. M. & Sciarrino, S. (1984) Cell Biol. Int. Rep. **8**, 79–85
4. Smith, B. D. & Niles, R. (1980) Biochemistry **19**, 1820–1825
5. Adams, S. L., Sobel, M. E., Howard, B. H., Olden, K., Yamada, K., de Crombrugghe, B. & Pastan, I. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3399–3405
6. Rowe, D. W., Moen, R. C., Davidson, J. M., Byers, P. H., Bornstein, P. & Palmiter, R. D. (1978) Biochemistry. **17**, 1581–1590
7. Avvedimento, V. E., Yamada, Y., Lovelace, E. & de Crombrugghe, B. (1981) Nucleic Acids Res. **9**, 1123–1131
8. Hata, R. I. & Peterkofsky, B. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2933–2937
9. Sandmeyer, S., Gallis, B. & Bornstein, P. (1981) J. Biol. Chem. **256**, 5022–5028
10. Peterkofsky, B. & Prather, W. (1986) J. Biol. Chem. **261**, 16818–16826
11. Marsilio, E., Sobel, M. E. & Smith, B. D. (1984) J. Biol. Chem. **259**, 1401–1404
12. Smith, B. D. & Marsilio, E. (1988) Biochem. J. **253**, 269–273
13. Smith, B. D. (1990) Ann. N.Y. Acad. Sci. **580**, 459–461
14. Hata, R. I., Ninomiya, Y., Nagai, Y. & Tskada, Y. (1980) Biochemistry **19**, 169–176
15. Lee, S.-T., Smith, B. D. & Greenspan, D. S. (1988) J. Biol. Chem. **263**, 13414–13418
16. Cedar, H. & Razin, A. (1990) Biochim. Biophys. Acta **1049**, 1–8
17. Adams, L. P. (1990) Biochem. J. **265**, 309–320
18. Doefler, W. (1989) in Nucleic Acids and Molecular Biology (Eckstein, F. & Lilley, D. M. J., eds.), vol. 3, pp. 92–119, Springer-Verlag, Berlin and Heidelberg
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (eds.) (1991), Current Protocols in Molecular Biology, Green Publishing Associates and Wiley-Interscience, New York
20. Boast, S., Su, M.-W., Ramirez, F. & Sanchez, M. (1990) J. Biol. Chem. **265**, 13341–13356
21. Dente, L., Sollazzo, M., Balderi, C., Cesareni, G. & Cortese, R. (1985) in DNA Cloning (Glover, D. M., ed.), vol. 1, pp. 101–107, IRL Press, Washington
22. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) Mol. Cell Biol. **2**, 1044–1051
23. Hall, S. V., Jacobs, P. E., Ringold, G. M. & Lee, F. (1983) J. Mol. Appl. Genet. **2**, 101–109
24. Bradford, M. M. (1967) J. Mol. Biol. **26**, 365–369
25. Kriek, E. (1974) Biochim. Biophys. Acta **155**, 177–203
26. Koehl, P., Burnouf, S. & Fuchs, R. P. P. (1989) J. Mol. Biol. **207**, 355–364
27. Schmidt, A., Setoyama, C. & de Crombrugghe, B. (1985) Nature (London) **314**, 286–289
28. McKeon, C., Ohkubo, H., Pastan, I. & de Crombrugghe, B. (1982) Cell (Cambridge Mass.) **29**, 203–210
29. McKeon, C., Pastan, I. & de Crombrugghe, B. (1984) Nucleic Acids Res. **12**, 3491–3502
30. Parker, M. I., Judge, K. & Gevers, W. (1982) Nucleic Acids Res. **10**, 2823–2833
31. Thompson, J. P., Simkevich, C. P., Holness, M. A., Kang, A. H. & Raghow, R. (1991) J. Biol. Chem. **266**, 2549–2556
32. Feinberg, A. P. & Bolgelstein, B. (1983) Nature (London) **301**, 89–92

Received 26 July 1991/9 October 1991; accepted 24 October 1991