Methylation of the $\alpha 2(I)$ collagen gene in chemically transformed rat liver epithelial cells

Barbara D. SMITH*† and Erika MARSILIO†

*Collagen Research Laboratory, Veterans Administration Outpatient Clinic, Boston, MA 02108, and

†Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, U.S.A.

W8 cells, 2-*N*-(acetoxyacetyl)aminofluorene-transformed rat liver epithelial-like cells, secrete no $\alpha 2(I)$ collagen chains. This paper reports the first demonstration of DNA methylation in the promoter-5' region of an $\alpha 2(I)$ collagen gene which occurs in W8 cells. Since inhibition of methylation by azacytidine induces transcription of the $\alpha 2(I)$ gene, DNA methylation of W8 $\alpha 2(I)$ promoter-5' region could contribute to altered collagen production in these cells.

INTRODUCTION

Collagen, a multigene family of connective tissue proteins, plays an important role in cellular differentiation and cell behaviour, as well as a structural role in maintenance of all tissue and organ systems except blood (for review see [1]). There are at least 12 types of collagens presently described which are coded by possibly 20 genes. The most abundant collagen in vertebrate tissue, designated type I collagen, is a triple helical molecule composed of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain. In some cases, three $\alpha 1(I)$ chains can form a molecule called $\alpha 1(I)$ trimer [2,3]. Most cells produce at least one type of collagen [4], while some cells have the ability to synthesize more than one type [5]. Rat liver epithelial cells in culture produce mainly type I collagen [6], as well as small amounts of type III [7] and type IV [8] collagen.

Since collagen is a modulator of cell behaviour, a change in collagen synthesis may be an important factor affecting transformed cells. Many studies have described altered collagen gene expression associated with the transformed phenotype [6,9–15]. Viral transformation of fibroblasts usually decreases collagen synthesis [6, 9–13]. In most cases, both type I collagen chains are co-ordinately decreased as a result of lower levels of translatable and/or hybridizable collagen mRNA in the transformed cells [10,11]. Decreased transcription of collagen mRNA may be responsible [12,13].

It is clear from several studies that chemical carcinogens also alter collagen gene expression [6,14,15]. Chemically-transformed NIH 3T3 cells exhibit changes not only in the amount of type I collagen, but also in the specific type of collagen expressed [14]. However, less information regarding the mechanism by which chemical carcinogens alter collagen synthesis is available.

To investigate this mechanism in epithelial-like cells further, we have studied a well characterized 2-*N*-(acetoxyacetyl)aminofluorene (AAF) transformed cell line, W8, and its parent cell line, K16, originally established by I. B. Weinstein [16]. Our previous results [6] showed that K16 cells secrete a normal type I collagen

molecule, whereas the W8 cells produce $\alpha 1(I)$ trimer. A 3' rat cDNA probe did not identify any stable translatable or hybridizable levels of pro $\alpha 2(I)$ mRNA in total mRNA isolated from W8 cells [17]. In this paper, we demonstrate that the gene for pro $\alpha 2(I)$ is present in W8 cells. The promoter-5' region of the pro $\alpha 2(I)$ gene in the W8 cells is methylated, whereas the same region in the K16 DNA is not. Inhibition of DNA methylation by azacytidine induces the pro $\alpha 2(I)$ transcription in the W8 cells.

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 calf serum, 1% penicillin G/streptomycin sulphate, and 1% glutamine as previously described [6,17]. In certain experiments, cells were grown in the presence of 10 μ M-azacytidine for 48 h.

DNA extractions

Cells were harvested in phosphate-buffered saline and lysed with lysing buffer (0.14 M-NaCl, 1.5 mM-MgCl₂, 0.5% NP-40, and 10 mM-Tris, pH 8.6). The nuclei were centrifuged at 10000 *g* for 20 min at 4 °C through a 24% sucrose, 1% NP-40 cushion. The pelleted nuclei were rinsed twice in the lysing buffer. Genomic DNA was extracted from the nuclei by standard methods as described by Maniatis *et al.* [18].

Electrophoresis and transfer of DNA to nitrocellulose

Genomic DNA (25–30 μ g) was digested by appropriate restriction enzymes using the manufacturers' recommended buffer. If two digestions were performed, the DNA was ethanol-precipitated after the first digestion and redissolved in the buffer for the second enzyme digestion. DNA was electrophoresed on 1% agarose horizontal gels in 0.089 M-Tris/borate, 0.089 M-boric acid, and 2 mM-EDTA, pH 8, buffer at 45 V for 16 h.

Abbreviation used: AAF, 2-*N*-(acetoxyacetyl)aminofluorene.

† To whom correspondence should be sent.

The gels were stained with ethidium bromide, treated and transferred to nitrocellulose membranes as described by Southern [19].

Recombinant bacterial plasmids

Rat collagen cDNA clones [20] (gifts from Dr. David Rowe, University of Connecticut Medical Center, Farmington, CT, U.S.A.) were used as described previously [17]. The p α 2R2 plasmid contains a 1500 bp insert encoding the C-terminal extension peptide of the pro α 2(I) molecule and extends into the 3' untranslated region of the mRNA.

In addition, a mouse genomic pro α 2(I) clone (pAZ1002) (gift from Dr. Benoit de Crombrughe and Dr. Azriel Schmidt, NIH, Bethesda, MD, U.S.A.) was used. The pAZ1002 plasmid has an 850 bp pro α 2(I) collagen gene fragment inserted within a pUC plasmid at the *Xho*I site. The pAZ1002 insert, extending from -108 to 741, encodes part of the promoter, the whole first exon and part of the first intron. Most of its sequence has been published [21]. Recombinant plasmids were propagated in *Escherichia coli* strain N38(C600 r⁻m⁻) in accordance with the National Institutes of Health guidelines for recombinant DNA research.

Preparation of DNA probes and hybridization

Bacteria were lysed and plasmids were extracted and partially purified [17,19], followed by equilibrium centrifugation in caesium chloride/ethidium bromide. The final preparations were electrophoresed on 1.4% agarose gels before and after restriction endonuclease digestion with *Pst*I or *Xho*I to exclude chromosomal DNA contamination and to confirm identity of the plasmids. Purified plasmids were nick-translated using the nick-translation kit of Amersham.

Nitrocellulose filters were pretreated for 24 h at 43 °C with 50% formamide, hybridized with ³²P-labelled probes (10⁶ c.p.m./lane) for 2–3 days at 43 °C, and washed three times as described previously [17].

After the nitrocellulose membranes were exposed to Kodak X-Omat AR film at -70 °C for varying amounts of time, the membranes were washed with 50% formamide in Tris/EDTA (1 mM-EDTA, 10 mM-Tris, pH 8) at 60 °C for 1 h to remove the radioactive probe. After confirming removal of hybridized probes by autoradiography, the filters were then rehybridized to another probe. All autoradiograms were scanned by an LKB densitometer equipped with an integrator to determine areas. Different exposures of the fluorograms were scanned to ensure a linear response range.

Nuclear run-off transcription assays

Separation of nuclei and cytoplasm of cells was performed according to Greenberg & Ziff [22]. Nuclear run-off assays were performed as described by Groudine *et al.* [23]. Each reaction consisted of 2 × 10⁷ nuclei in 200 μ l of reaction buffer. The nuclear suspension was incubated at 30 °C for 30 min.

The purified RNA [23] (5 × 10⁶ c.p.m.) was hybridized to nitrocellulose filters containing immobilized plasmid DNAs (10 μ g) blotted with a Schleicher and Schuell Slot Blot Apparatus according to the manufacturer's directions. Hybridization was carried out for 36 h at 65 °C in 600 mM-NaCl, 0.2% SDS, 10 mM-EDTA, 10 mM-Tes, pH 7.4 and washed with several changes of 2 × SSC at 65 °C. The filters were then incubated with 10 μ g of

RNAase A/ml (Worthington) at 37 °C for 30 min and washed again with 2 × SSC. After air drying, the filters were exposed to X-ray film.

RESULTS

Since there has been no evidence of pro α 2(I) protein [6] or mRNA [17] in the W8 cells, we examined the pro α 2(I) gene structure. Genomic DNA from K16 and W8 cells was extracted and restriction digested by a series of restriction enzymes (*Eco*RI, *Xba*I, *Pst*I, *Hind*III, *Pvu*II, *Hae*III, *Hinf*I, *Cl*aI, *Bgl*qII, and *Sma*I). The restriction fragments were Southern blotted from agarose gels and hybridized to cDNA 3' and genomic 5' DNA probes. There were no differences in the size or number of the restriction fragments on any Southern blots comparing K16 with W8 DNA.

Several enzymes that are sensitive to methylation did produce different patterns by Southern analysis. Therefore, we examined methylation of the pro α 2(I) gene by digesting genomic DNA from both K16 and W8 cells with the isochizomers *Hpa*II or *Msp*I. *Hpa*II will not cleave when the internal C residue is methylated, whereas *Msp*I is active regardless of methylation. *Eco*RI digestion was performed prior to digestion with the isochizomers. A Southern blot of these restriction digests is shown in Fig. 1. DNA from W8 and K16 cells digested with *Eco*RI contained one 2000 bp fragment that hybridized to 5' pro α 2(I) genomic probe (Fig. 1a, lanes 1 and 2). The *Eco*RI/*Msp*I digest of both K16 and W8 DNA resulted in one 600 bp fragment (Fig. 1a, lanes 3 and 4). However, the *Eco*RI/*Hpa*II digest of K16 DNA was considerably different from the same digest of the W8 DNA (Fig. 1a, lanes 5 and 6). The *Eco*RI/*Hpa*II digest of K16 DNA resembled the *Eco*RI/*Msp*I digest with a major fragment of 600 bp. Only about 13% of the hybridizable counts were found in the undigested 2000 bp fragment suggesting low levels of methylation at *Msp*I/*Hpa*II sites in K16 DNA. However, 35% of the hybridizable counts were found in the 2000 bp fragment of the *Eco*RI/*Hpa*II digest of W8 cells (Fig. 1a, lane 6) and 55% were found in two other fragments at 1000 and 850 bp suggesting hypermethylation of the gene at several *Hpa*II sites. Only 10% of the hybridizable counts were found in the 600 bp completely digested fragment. Using mouse sequence data, a fragment is predicted containing three methylated *Msp*I sites at approx. 1000 bp and two sites at 850 bp as shown in Fig. 1b.

This same blot was rehybridized to the 3' pro α 2(I) rat cDNA probe as seen in Fig. 2. The *Eco*RI digest resulted in one large fragment, approx. 6000 bp (Fig. 2, lanes 1 and 2), that disappeared with additional *Msp*I digestion producing a 2000 bp fragment (Fig. 2, lanes 3 and 4). The two lower fragments (1000 bp and 740 bp) remained stable under all conditions. The *Eco*RI/*Hpa*II digest of W8 and K16 DNA (Fig. 2b, lanes 5 and 6) resulted in some undigested *Eco*RI fragments and two additional fragments (approx. 4900 and 4600 bp). These results indicate that the 3' region of the pro α 2(I) gene is methylated in both cell types. There was a 3-fold increase in undigested fragments in the K16 DNA compared with the W8 DNA, suggesting that the K16 3' region of the pro α 2(I) gene was slightly more methylated compared with the same region in the W8 gene. Therefore, the degree of methylation in the 3' region of the gene did not correlate with the absence of transcription.

Fig. 1. Methylation status of the promoter-5' region (5' α 2) of the pro α 2(I) gene in K16 (K) or W8 (W) cells

(a) Total DNA from K16 (K) and W8 (W) cells was cleaved with *Eco*RI (*Eco*) alone or in combination with *Msp*I (+*Msp*) or *Hpa*II (+*Hpa*). DNA was fractionated by electrophoresis on a 1% agarose gel, Southern blotted and hybridized with mouse genomic promoter-5' pro α 2(I) probe, pAZ1002 (5' α 2). (b) Diagram of methylation sites (M) in the promoter-5' region of the pro α 2(I) gene. Line (1) represents the probe from pAZ1002. Line (2), represents the promoter-5' region of the α 2(I) gene. The arrow locates the start of transcription of the first exon shown as a heavy line. Lines (3)–(7) represent fragments generated by *Hpa*II digests of partially methylated DNA shown with the approximate base pairs for each one. Line (7) designates the base pairs with the mRNA start site as 0. The restriction enzymes are designated as follows: H, *Hpa*II; E, *Eco*RI; S, *Sma*I; M, methylated *Hpa*II site.

The Southern blot shown in Fig. 2 was also hybridized to a 5' β -tubulin cDNA probe (gift from Dr. S. Farmer) and a 5' mouse pro α 1(I) genomic probe (gift from Dr. R. Jaenisch). A similar blot was hybridized to the rat 3' cDNA pro α 1(I) and the α 2R2 probe. The results for the tubulin and the pro α 1(I) gene (not shown) were similar to the hybridization pattern obtained with the pro α 2(I) cDNA probe. The *Eco*RI/*Hpa*II digest indicated that these areas of the β -tubulin and the pro α 1(I) genes were methylated and the K16 DNA was slightly more methylated than the W8 DNA. This suggests a general hypomethylation in the W8 genome with the exception of hypermethylation in the promoter-5' region of the pro α 2(I) gene.

Since differences were noted in the methylation pattern between cells in the 5' region of the pro α 2(I) gene, preliminary experiments were performed using azacytidine to reduce DNA methylation. Nuclear transcription assays demonstrated that nuclei from azacytidine-treated W8 cells could transcribe pro α 2(I) mRNA (Fig. 3, third panel). No pro α 2(I) transcription could be detected in W8 cells. The level of collagen transcription was much lower than histone or actin (results not shown). Although this experiment must be interpreted with caution because of possible secondary effects of this drug, the results strongly suggest that methylation is at least partially responsible for inhibiting transcription of the W8 pro α 2(I) gene.

DISCUSSION

In our previous papers [6,17], we showed that W8 cells produced a homotrimer of pro α 1(I) chains. No pro α 2(I) chains were secreted by W8 cells [6]. In addition, there were no stable mRNAs for pro α 2(I) in W8 cells as judged by translation studies *in vitro* or by hybridization to rat cDNA procollagen probes (p α 1R1 and p α 2R2) [17]. The parent cell line, K16, did contain pro α 2(I) RNA.

This paper establishes that chemically-transformed rat liver epithelial W8 cells contain a pro α 2(I) gene without any large insertions, deletions or rearrangements as judged by Southern blot analysis. The pro α 2(I) gene contains a hypermethylated 5' region compared with the pro α 2(I) gene from the parent K16 cell line (Fig. 1). This hypermethylation was specific for the 5' region of the W8 pro α 2(I) since increased hypermethylation was not detected in the 3' region (Fig. 2) of the W8 α 2(I) gene, α 1(I) gene, or tubulin gene. This increased DNA methylation of the W8 5' region of the α 2(I) gene correlates with the previously described lack of pro α 2(I) protein [6] and steady state mRNA [17]. Treatment of W8 cells with azacytidine, an inhibitor of DNA methylation, allowed α 2(I) transcription in W8 cells (Fig. 3) suggesting that the methylation in the W8 α 2(I) promoter inhibited transcription.

Transcription of many genes is regulated by methylation at specific sites in the promoter or 5' region [24]. The

Fig. 2. Methylation status of the 3' region (3'α2) of proα2(I) gene in K16 (K) and W8 (W) cells

Same Southern blot as Fig. 1 rehybridized with rat cDNA proα2(I) probe, pα2R2.

methylation of the W8 proα2(I) promoter is the first demonstration that this portion of the proα2(I) gene can be methylated. Others [25, 26] have demonstrated that the 5' region of the chick proα2(I) gene was not methylated in DNA from a variety of tissues, from chicken embryo fibroblasts before and after viral transformation or from chick chondrocytes during modulation of type I collagen production. There seemed to be inhibition of methylation in this area of the gene whether or not the chick proα2(I) gene was expressed. Methylation of the 5' region of other collagen genes has not been reported as yet.

Methylation of the 3' region of type I and type II collagen genes has been examined previously [25,26]. McKeon *et al.* [25] found that the 3' region of the chick proα2(I) gene was methylated. There were differences in the amount of methylation between cell types, but the changes in methylation did not correlate with changes in transcription. Fernandez *et al.* [26] extended these findings to chondrocytes and to the type II collagen gene. In both these papers, methylation patterns were not altered when cells in culture were induced to change transcription levels of collagen by several agents (RSV infection, bromodeoxyuridine, or vitamin A). On the other hand, it has been demonstrated [27] that human WI-38 fibroblasts, infected by SV40 virus, did have increased methylation in the 3' region of the proα1(I) and the proα2(I) gene. Hypermethylation correlated with an absence of type I collagen mRNA. However, azacytidine data did not support methylation as a mechanism [28]. Taken together the data suggest that methylation in the 3' region of collagen genes varies considerably and does not correlate with changes in transcription.

Fig. 3. Nuclear transcription assays with nuclei from K16, W8, and azacytidine-treated W8 cells

Nuclei (2×10^7) from K16, W8, and azacytidine-treated W8 cells (aza W8) were incubated with radiolabelled UTP. Purified RNA from each nuclei (5 ± 10^6 c.p.m. per blot) were hybridized to nitrocellulose containing immobilized probes (10 μ g), pα1R2 (α1), pα2R2 (α2), histone (HIS) and pUC (PUC) plasmid as control.

The chemical used to transform W8 cells, AAF, forms an adduct at the C-8 position of G residues. There has been controversy regarding the methylation state of DNA after AAF treatment [29]. Studies demonstrate both hypermethylation and hypomethylation of the DNA in cultured cells [29]. These studies examine overall methylation of the genome so that hypomethylation or hypermethylation of small areas in genes could not be detected. Our studies examining individual areas of genes suggests an overall hypomethylation of the W8 genome compared with the parent cell genome with a hypermethylation of the α2(I) promoter-5' region.

Our current hypothesis is that under normal circumstances methylation of the collagen promoter is rigorously inhibited in CG rich regions. Therefore, alterations in gene transcription during differentiation occurs by other mechanisms. However, outside influences that alter DNA structure can cause methylation in a critical area that inhibits transcription. This could possibly occur in certain areas of DNA that are more sensitive to disruption.

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