DNA Methylation and Collagen IV Gene Expression in F9 Teratocarcinoma Cells*

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Although undifferentiated F9 teratocarcinoma cells express low levels of mRNAs for collagen IV, previous transient transfection experiments using an αI(IV) collagen gene promoter-enhancer-CAT construct revealed a high level of transcriptional activity in these cells. In this report, we find that when this construct is introduced stably into undifferentiated F9 teratocarcinoma cells, expression does not occur unless the cells are treated with retinoic acid and dibutyryl-cAMP. Such activation mimics the endogenous gene activity. Treatment of the cells containing the integrated construct with 5-azacytidine, an agent which prevents DNA methylation, also activates transcription and acts synergistically with retinoic acid and cAMP. Analysis of DNA isolated from F9 teratocarcinoma cells revealed that there was a specific demethylation of the DNA within the 5'-flanking region of the collagen IV genes following treatment with retinoic acid and cAMP. These results suggest that during differentiation, DNA demethylation may play an important role in transcriptional regulation of the collagen IV genes.

Collagen IV, the major structural component of basement membranes, is composed of two α1(IV) and one α2(IV) chains. The genes for the two polypeptides exist in a head to head arrangement on opposite strands of DNA, separated by 130 base pairs, in both the mouse (Burbelo et al., 1988; Kaytes et al., 1989) and the human (Poschl et al., 1988; Soininen et al., 1988). The close proximity and arrangement of the two collagen IV genes on the same chromosome distinguishes these genes from those of other collagen types which are dispersed throughout the genome (Solomon et al., 1985). In addition, both the α1(IV) and α2(IV) chain genes share a bidirectional promoter which utilizes an enhancer element within the first intron of the α1(IV) collagen gene for efficient transcriptional activity (Burbelo et al., 1988).

The transcriptional regulation of these and other basement membrane component genes is often studied in F9 teratocarcinoma stem cells. These cells differentiate into parietal endoderm-like cells in the presence of both retinoic acid and dibutyryl-cAMP (Strickland et al., 1980) and show a striking coordinate and increased synthesis of basement membrane proteins including collagen IV (Kleinman et al., 1987; Kurkiinen et al., 1983; Marotti et al., 1985; Wang and Gudas, 1983) and laminin (Carlin et al., 1983; Kleinman et al., 1987; Wang and Gudas, 1983). Thus, F9 teratocarcinoma cells provide a model system for studying the regulation of these genes. Induction of the mRNA for these genes requires approximately 18-24 h and is blocked by the addition of cycloheximide indicating that protein synthesis is required (Gudas and Wang, 1986).

The promoter-enhancer constructs for the α1(IV) collagen chain gene are not promiscuously expressed in all cells, but rather only in cells producing the protein (Killen et al., 1988). Undifferentiated F9 teratocarcinoma cells express very low levels of collagen IV; however, they show a high level of transcriptional activity of the promoter-enhancer-CAT construct in transient assays (Killen et al., 1988). One possible explanation is that undifferentiated F9 cells contain the trans-acting factors that activate both the promoter and enhancer regions for collagen IV genes, but do not bind to the regulatory regions of the endogenous gene because of their methylation state and/or chromatin structure. To investigate this possibility, we have integrated stably α1(IV) chain promoter-enhancer CAT constructs into undifferentiated F9 teratocarcinoma cells and found that they are not transcribed in these cells. Following treatment of the cells with retinoic acid and dibutyryl-cAMP, however, there was a marked stimulation of transcriptional activity. We have also found that the 5'-flanking DNA of the α1(IV) gene was demethylated during differentiation of F9 cells. These results indicate that the methylation state and/or chromatin structure of the collagen IV genes, rather than the production of trans-acting factors, may play a key role in the activation of collagen IV genes in this system.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DNA Transfections**—The F9 teratocarcinoma cells were maintained in culture in the undifferentiated state as described previously (Killen et al., 1988). Differentiation of the cells to parietal endoderm-like cells was initiated by addition of all-trans retinoic acid (10^-7 M) plus dibutyryl-cAMP (10^-3 M) (Strickland et al., 1980). The α1(IV) promoter-CAT construct (p47A) and the α1(IV) promoter-enhancer-CAT construct (p48) have been described previously (4). Transient transfection into undifferentiated F9 teratocarcinoma cells was performed as described previously (Burbelo et al., 1988). Briefly, cells (250,000) were transfected with 5 μg of plasmid DNA by the calcium phosphate method (Graham and Van der Eb, 1973). Eighteen hours later, the medium was changed, and the cells were incubated either in normal medium or in the presence of dibutyryl-cAMP and retinoic acid. Forty eight hours after the medium was changed, the cells were harvested and assayed for CAT activity (Gorman et al., 1982).

For stable transfection, undifferentiated F9 cells were cotransfected with 2 μg of the CAT plasmids and 18 μg of RSVneo using the calcium phosphate method (Graham and Van der Eb, 1973). Colonies

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expressing the neo gene were isolated based on their resistance to the antibiotic G418. Resistant colonies were pooled (>200/transfection), expanded in mass cultures in the presence of G418 (350 μg/ml), and used for experiments. Some cells (250,000) were treated for 48 h with retinoic acid and dibutyryl-cAMP and then assayed for CAT activity. Other cells were treated with 5-azacytidine (10^{-6} M) for 24 h after which medium was changed and maintained drug-free for 24 h.

Individual clones of cells containing the stably transfected α1(IV) chain gene promoter-enhancer-CAT construct were also obtained by the limited dilution method. CAT activity was determined in cells which had been cultured with and without retinoic acid and dibutyryl-cAMP for 48 h. The copy number of the collagen IV promoter-enhancer-CAT construct in the individual clones was determined by dot hybridization using a Real fragment from the coding region of the CAT gene (Brinster et al., 1985). CAT activity is expressed as raw counts or as a percentage of total chloramphenicol converted to acetylated derivatives.

**DNA Methylation Pattern**—High-molecular weight cellular DNA was prepared from both undifferentiated and differentiated F9 teratocarcinoma cells by the procedure of Gross-Bellard et al. (1973). Aliquots (25 μg) of DNA were digested with HindIII, XhoI, and/or MspI restriction endonucleases (5 units/μg of DNA) for 18 h at 37°C. DNA samples were electrophoresed on 1% agarose gel and transferred to nitrocellulose by the method of Southern (1975). The probe to the 5’-end of the collagen IV genes was a 0.72-kb XbaI-NcoI DNA fragment containing the bidirectional promoter region, part of the first exon of the α1(IV) collagen, and the first exon, intron, and 20 base pairs of untranslated sequence from the second exon of the α2(IV) collagen gene. This fragment was labeled by the random prime method (Feinberg and Vogelstein, 1982) and used as probe.

**RESULTS**

Previously, we have shown that the DNA segment between +2.7 and +5.0 kb in the first intron of the α1(IV) collagen gene contains enhancer activity which is essential for the cell-specific activation of both the α1(IV) and α2(IV) collagen genes (Burbelo et al., 1988; Killen et al., 1988). CAT activity of transiently transfected F9 teratocarcinoma cells is shown in Fig. 1A. A β-actin CAT construct, which served as control, displayed a similar high level of activity in both cell types regardless of whether the cells were treated with retinoic acid plus cAMP or not (Fig. 1A, lanes C and D). The promoter-CAT construct for the α1(IV) collagen gene was found to be inactive under both conditions as shown previously (Fig. 1A, lanes A and B). The α1(IV) chain gene promoter-enhancer-CAT construct exhibited a high level of activity in both cell types treated with cAMP alone or β-actin-CAT and in F9 cells cotransfected with RSVneo. The percent acetylation is shown below the corresponding lane.

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**FIG. 1. A, transient transfection of the α1(IV) chain gene-CAT plasmids in F9 teratocarcinoma cells.** Undifferentiated F9 cells were transfected with 5 μg of plasmid DNA. After 18 h, the DNA was removed and the cells were untreated (−) or treated with dibutyryl-cAMP and retinoic acid (+). 48 h later, CAT activity was determined. Lanes A and B represent the α1(IV) promoter-CAT-construct − and +; lanes E and F represent the α1(IV) promoter-enhancer-CAT construct − and +; and lanes C and D are β-actin-CAT construct − and +. The percent acetylation is shown below the corresponding lane.

To evaluate the possibility that the methylation state of the DNA and/or chromatin structure was important in transcriptional regulation of the collagen IV genes, we established lines of F9 cells containing integrated copies of α1(IV) chain gene-CAT constructs. Undifferentiated F9 teratocarcinoma cells were co-transfected with either the α1(IV) promoter-CAT or α1(IV) promoter-enhancer-CAT construct and RSVneo. After 4 weeks, pooled cells (from approximately 200 G418-resistant colonies) were examined for CAT gene expression before and after treatment with retinoic acid and dibutyryl-cAMP. The control β-actin CAT construct again displayed a similar high level of activity independent of whether the cells were induced to differentiate with retinoic acid and cAMP (Fig. 1B, lanes C and D). The α1(IV) promoter construct lacking the enhancer was inactive when integrated stably (Fig. 1B, lanes A and B), as found in the transient transfection experiments. The promoter-enhancer-CAT construct was not active in undifferentiated F9 cells (Fig. 1B, lane E); however, it could be activated by treatment of cells with retinoic acid plus cAMP (Fig. 1B, lane F). Dibutyryl-cAMP alone was ineffective in activating the collagen IV constructs (Fig. 2, lane B), whereas retinoic acid induced transcription (Fig. 2, lane C) and acted synergistically with cAMP (Fig. 2, lane D). The addition of a calcium-phosphate DNA precipitate to the undifferentiated cell constructs did not result in an activation of CAT activity (data not shown).

This ruled out the possibility that the collagen IV constructs were being activated by the transfection procedure, as has been shown for other genes (Fine et al., 1988).

Individual clones from the pooled cells containing stably
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Fig. 2. Effect of CAMP and retinoic acid on the stably integrated collagen IV promoter-enhancer-CAT constructs in F9 teratocarcinoma cell. Pooled undifferentiated F9 cells containing the stably integrated α1(IV) promoter-enhancer-CAT constructs were untreated (lane A), treated only with CAMP (lane B), treated only with retinoic acid (lane C), or treated with both retinoic acid and CAMP (lane D). The level of CAT expression shown here parallels the endogenous levels of α1(IV) chain mRNA in F9 cells treated in this manner. The percent acetylation is shown below the corresponding picture of the autoradiograph. Data represent greater than three replications.

Table I
Characterization of clones containing the α1(IV) promoter-enhancer-CAT construct

<table>
<thead>
<tr>
<th>Clone</th>
<th>CAT copy number</th>
<th>Fp + CAT activity</th>
<th>Fp CAT activity</th>
<th>Increase following differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9</td>
<td>1,921</td>
<td>34,617</td>
<td>18</td>
</tr>
<tr>
<td>B</td>
<td>48</td>
<td>5,090</td>
<td>30,130</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>11</td>
<td>435</td>
<td>15,095</td>
<td>35</td>
</tr>
<tr>
<td>F</td>
<td>12</td>
<td>6,987</td>
<td>193,609</td>
<td>28</td>
</tr>
<tr>
<td>H</td>
<td>14</td>
<td>1,036</td>
<td>11,554</td>
<td>11</td>
</tr>
<tr>
<td>I</td>
<td>48</td>
<td>4,133</td>
<td>317,775</td>
<td>77</td>
</tr>
<tr>
<td>K</td>
<td>9</td>
<td>435</td>
<td>331,130</td>
<td>762</td>
</tr>
</tbody>
</table>

The integrated collagen IV promoter-enhancer-CAT constructs were also analyzed for CAT gene expression and copy number (Table I). Nine of eleven clones were found to have the CAT gene, whereas only two clones contained just the RSV neo gene. All nine clones showed a marked increase in CAT activity following retinoic acid and dibutyryl-cAMP treatment suggesting that the site of integration was not critical for the activation of these constructs. However, the number of integrated copies did not correlate directly with the level of activity observed following retinoic acid and dibutyryl-cAMP treatment.

The difference in transcriptional activity seen between transient and stable transfections could be due to differences in the methylation state in the enhancer and/or promoter. The pooled clones containing the integrated promoter-enhancer-CAT construct were therefore treated with 5-azacytidine, which blocks DNA methylation of replicating cells. 5-Azacytidine treatment increased transcription of the collagen IV construct (Fig. 3, lane C) and had a marked synergistic effect when added with retinoic acid and cAMP (Fig. 3, lane D).

The methylation pattern of the 5′-end of the collagen IV genes was analyzed in both undifferentiated and differentiated F9 cell DNA using the two isoschizomers MspI and HpaII. Both MspI and HpaII recognize the sequence CCGG, although MspI will not cut this sequence when the 5′ cytosine is methylated, whereas HpaII will not cut when either cytosine is methylated. No difference in the methylation pattern between the undifferentiated and differentiated F9 cells was detected using MspI (Fig. 4, lanes 1 and 9). The methylation pattern of the 5′-end of the collagen IV genes during F9 teratocarcinoma cell differentiation. DNA was isolated from both undifferentiated or differentiated F9 cells (14). DNA was digested with the various restriction enzymes, electrophoresed, and blot-hybridized with a probe to the 5′-end of the collagen IV genes as described under "Experimental Procedures." The size of the fragments are shown in kilobases. Differentiated F9 cell DNA is represented by lanes 1–6, whereas undifferentiated F9 cell DNA is represented by lanes 7–12. Lanes 1 and 9 were digested with MspI; lanes 2 and 8 were digested with MspI/HindIII; lanes 3 and 7 were digested with MspI/XhoI; lanes 4 and 12 were digested with HpaII; lanes 5 and 11 were digested with HpaII/HindIII; and lanes 6 and 10 were digested with HpaII/XhoI.
DNA methylation appears to be an important mechanism which regulates gene expression. With some exceptions, the transcriptional activation of certain genes correlates with the hypomethylation of certain CpG residues in the regulatory elements of cells which express the genes (see Bird, 1986; Cedar, 1988). Using direct genomic sequencing of regulatory regions, specific CpG residues have been identified which are methylated in nonexpressing cells, but are demethylated when the gene is actively transcribed (Becker et al., 1986; Saluz et al., 1986). Furthermore, 5-azacytidine, a potent demethylating agent, has been shown to activate cellular genes in replicating cells by inhibiting the conservative methylation reaction (Charache et al., 1983; Desimone et al., 1983; Jones, 1985; Jones and Taylor, 1980; Ley et al., 1982).

The expression of collagen IV appears to be highly regulated both spatially and temporally during development. The expression of both the α(1)IV and α(2)IV chain genes requires both a bidirectional promoter and a shared enhancer located in the first intron of the α(1)IV gene (Burbelo et al., 1988). Transient transfection experiments with a collagen IV promoter-enhancer-CAT construct generally reveal a cell-specific expression in cells, which correlates with their ability to produce collagen IV (Killen et al., 1988).\(^2\) One exception to this cell-specific expression is found in undifferentiated F9 cells, which show a high level of activity in the transient assay but show very low levels of collagen IV mRNA. In contrast, the integration of the collagen IV promoter-enhancer-CAT constructs in undifferentiated cells extinguishes expression, whereas treatment of these cells with retinoic acid induces a high level of CAT expression. The inactivation of the collagen IV promoter-enhancer-CAT construct by stable integration is not novel, since other foreign genes introduced into both cells and transgenic mice have also been shown to become methylated and inactive upon genomic integration (Jaenisch et al., 1985; Jahner et al., 1982; Palmer et al., 1982; Steward et al., 1982). Unlike these genes, the stably integrated collagen IV promoter-enhancer-CAT construct was able to be reactivated upon treatment with retinoic acid, paralleling the induction of endogenous levels of collagen IV mRNA. Although 5-azacytidine has many effects including the alteration of differentiation, the activation of the stably integrated collagen IV CAT constructs by this agent suggested that the methylation state might be important in regulating collagen IV genes during differentiation by altering the methylation state of the promoter.

Analysis of the methylation state of the endogenous collagen IV genes revealed a higher level of methylation in the 5'-flanking region of the α(1)IV collagen gene corresponding to the third intron of the α(2)IV collagen gene in undifferentiated, undifferentiated F9 cells compared to expressing, differentiated F9 cells. Methylation analysis using a probe covering the active region of the collagen IV enhancer within the first intron of the α(1)IV revealed no differences between undifferentiated and differentiated F9 cells, although sequence analysis revealed very few MspI/HpaII sites (data not shown). Since not all CpGs are accessible to methylation sensitive restriction analysis, it is possible that greater changes in the methylation state of the bidirectional promoter and shared enhancer exist, which cannot be detected with this methodology. For example, the mouse and human collagen IV bidirectional promoter is 23 and 30% CpG-rich, respectively, yet contains no 5'-CCGG-3' sites for methylation analysis by MspI/HpaII. Sequence analysis of the collagen IV bidirectional promoter shows a large dyad of symmetry containing a sequence which resembles a potential retinoic acid-responsive sequence (Umesono et al., 1988). One possibility is that during F9 teratocarcinoma cell differentiation, a retinoic acid-receptor complex might have a greater affinity for this binding site than the DNA methylase resulting in the general hypomethylation of the DNA in the promoter region. These results suggest that retinoic acid may mediate some of its effects by altering the methylation state. Two other hormones, estrogen (Jost et al., 1984; Saluz et al., 1986) and glucocorticoid (Mermod et al., 1983; Saluz et al., 1986), have also been shown to both induce gene activation via DNA demethylation.

The mechanism by which the methylation state controls collagen IV gene expression is intriguing. It is possible that DNA methylation may produce inactive chromatin structures (Groudine et al., 1981; Michalowsky and Jones, 1989). For example, in vitro methylation of DNA prior to integration has been shown to block expression (Busslinger et al., 1983; Keshet et al., 1985; Stein et al., 1982; Yisraeli et al., 1986) and product DNAase I-insensitive structures characteristic of inactive genes (Keshet et al., 1986). Furthermore, in the case of the human dihydrofolate reductase promoter, the methylation state and chromatin structure of the regulatory DNA was found to be an intrinsic property of the DNA (Shimada et al., 1987). Thus, methylation of the sites within the 5'-flanking region of the α(1)IV collagen gene in undifferentiated F9 teratocarcinoma cells may produce transcriptionally inactive chromatin structures at the 5'-end of the collagen IV genes. Alternatively, methylation of certain cis-acting elements within the promoter and/or enhancer, not detected by the methylation sensitive enzymes MspI/HpaII, may interfere with the binding of some trans-acting factors. As has been recently shown in vitellogenin gene expression, a ubiquitous factor (NH-2) displayed altered binding depending on the methylation state (Feaver et al., 1987). The presence of a CAMP-like responsive element within the collagen IV enhancer may also be a potential target of methylation regulated control. CpG methylation of the CAMP-responsive

\(^2\) P. D. Burbelo, unpublished data.
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Element has been shown to abolish specific factor binding and transcriptional activation (Iguchi-Ariga and Schaffner, 1989). Future experiments using genomic sequencing will be useful in identifying whether sites within the promoter and/or enhancer of the collagen IV genes show altered methylation during F9 teratocarcinoma cell differentiation.

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