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Loss of type I procollagen gene expression in SV40-transformed human fibroblasts is accompanied by hypermethylation of these genes

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ABSTRACT

Transformation of human lung fibroblasts (WI-38) by Simian Virus 40 (SV40) resulted in a decline of 25-30% in the amount of secreted collagen. The collagen produced by the transformed fibroblasts contained no type I collagen (i.e. α1(I) and α2 chains), which was the major collagen component produced by untransformed fibroblasts. Measurement of the procollagen mRNA levels by dot hybridization with nick-translated procollagen-cDNA clones showed that the absence of type I collagen was due to the absence of α1(I) and α2 procollagen mRNAs. This result was confirmed by hybridization of cDNA to total RNA with southern blots of the procollagen clones. To clarify the mechanism by which type I procollagen gene transcription is abolished in transformed cells, the methylation patterns of the α1(I) and α2 procollagen genes in normal and SV40-transformed fibroblasts were compared, using the chicken α1(I) and α2 procollagen-cDNA clones as probes. Methylated sites were detected by means of the restriction endonuclease isoschizomers HpaII and MspI. Methylation of the procollagen α1(I) and α2 genes was increased in the SV40-transformed fibroblasts, concurrently with the loss of type I collagen synthesis. DNA methylation may thus contribute to altered regulation of gene expression upon cell transformation.

INTRODUCTION

Neoplastic transformation of normal fibroblasts results in significant changes in the synthesis of various macromolecules. Some of these alterations affect the formation of extracellular matrix components. Thus increased levels of hyaluronic acid (1,2) and decreased levels of proteoglycans (3,4,5), fibronectin (5) and collagen have been observed. Collagen is perhaps the most extensively studied matrix component in both virally (7-21) and chemically (10,19,22) transformed fibroblasts. Transformation is accompanied by loss of anchorage dependence and density-dependent inhibition of growth; this suggests that decreased synthesis of some extracellular matrix components is functionally related to neoplastic transformation. Thus Sandmeyer et al (19) found that the severity of transformation in a series of cells was directly proportional to the degree by which collagen synthesis was decreased. Altered gene expression following on transformation furnishes a

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system to study the regulation of affected genes and also the mechanism by which specificity in this process is achieved. Our previous studies (15) as well as those of others (16,17) have shown that decreased collagen gene expression is generally due to decreased collagen gene transcription.

The collagens represent a family of at least nine genetically distinct alpha chains, with normal fibroblasts producing 75-90% type I collagen (23-25). In order to study the abolition of type I procollagen gene expression in SV40-transformed cells, we used pro α1(I) and pro α2 cDNA clones. Possible explanations for the abolition of type I procollagen gene expression could include (i) deletion of regulatory or coding sequences as in the case of the thalassaemias, (ii) production of an inhibitor specific for type I procollagen gene transcription, or (iii) modification of the type I procollagen genes, resulting in their inactivation. It is also possible that an inhibitor could recognise modified DNAs. Several studies have implicated DNA methylation in the regulation of gene expression. Amongst the systems studied are the globin genes (26-31), the ovalbumin and conalbumin genes (32,33) and the genes for immunoglobulins (34-36), metallothionein-1 (37) and delta-crystallins (38). These studies have shown hypermethylation of specific genes in tissues in which the gene products are not made. Approximately 90% of the 5-methylcytosine in eukaryotic DNA occurs in the dinucleotide CpG (39) and these sites can be detected by combinations of restriction endonucleases which are respectively sensitive and insensitive to methylated sites in their recognition sequences (40,41).

Our studies show that SV40-transformed WI-38 human lung fibroblasts totally shut off their production of type I collagen with the continued synthesis of a minor collagen component. This "switch" in collagen gene expression is accompanied by hypermethylation of the type I procollagen genes in the transformed fibroblasts.

MATERIALS AND METHODS

WI-38 human lung fibroblasts and SV40-transformed counterparts were gifts from Drs Gary and Janet Stein (University of Florida, USA), the α1(I) (pCg 54) and α2 (pCg 45) procollagen cDNA clones were gifts from Dr Helga Boedtker (Harvard Medical School, USA), and avian myeloblastosis virus reverse transcriptase was supplied by Dr J.W. Beard (Life Sciences Inc., Florida, USA). Restriction enzymes were obtained from Boehringer Mannheim or from Bethesda Research Laboratories, Maryland, USA. All chemicals were analytical (Merck or BDH) and solutions were rendered sterile and RNAse free by treatment with

0.1% diethylpyrocarbonate and autoclaving.

Collagen Synthesis

Cells were plated at 2 x 10^6 per 150 cm² flask in BME containing 10% foetal calf serum. After seven days, the cells were labelled with 5 µCi/ml of ^{3}H proline (Amersham, 25 Ci/m mole) in the above medium containing 50 µg/ml ascorbic acid and 50 µg/ml β-aminopropionitrile. The medium was removed and centrifuged at 10 000 x g for 5 minutes to pellet cellular debris. The supernatant was adjusted to pH 7.5 and NaCl added to a final concentration of 5 M to precipitate the collagens. After stirring at 4°C for six hours, the precipitate was pelleted at 30 000 x g for fifteen minutes at 4°C, dissolved in 10 mM Tris-HCl pH 7.5 and dialysed against the same buffer for 16 hours at 4°C to remove excess NaCl. The cells in the flasks were trypsinized and counted in a Coulter Counter, so that aliquots corresponding to the same amount of cellular material could be loaded onto polyacrylamide gels. Samples to be collagenase digested were brought to 50 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$ and 2.5 mM N-ethylmaleimide, as described by Peterkofsky and Diegelman (43). Samples to be digested with pepsin were dissolved in 200 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$, and incubated at 15°C for eight hours with 100 µg/ml pepsin. Foetal calf skin collagen was isolated as described by Piez (44). The samples were analysed on 5-11% gradient SDS-polyacrylamide gels. After staining with 0.25% Coomassie blue and destaining in 10% acetic acid, gels were soaked in 1 M salicylate for 30 minutes, dried and exposed to Du Pont Cronex 4 X-ray film.

DNA Isolation

Cells were grown to confluency in 150 cm² flasks in BME containing 10% foetal calf serum. Cells were scraped off into ice-cold, phosphate-buffered saline with a rubber policeman. Nuclei were prepared as previously described (45,46): briefly, the cells were suspended in RSB-buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$) containing 1% Triton X-100 and homogenized with ten strokes of the tight pestle of a Dounce homogenizer. Nuclei were pelleted at 1000 x g for 5 minutes at 4°C. The pellet was resuspended in RSB-Triton and processed as above. The pellet was dissolved in double-strength SSC (SSC = 0.15 M NaCl, 0.015 M Na Citrate), SDS was added to 1% and proteinase K to 100 µg/ml, and the mixture was then incubated at 50°C for 1 hour. Deproteinisation was effected by repeated extraction with an equal volume of phenol (saturated with 10 mM Tris-HCl pH 7.5, 1 mM EDTA), followed by two chloroform : isoamyl-alcohol (24:1) extractions. DNA was precipitated by the addition of 1/10 volume of 5 M NaCl and 2½ volumes of 96% ethanol.

After overnight precipitation at -20°C, the DNA was wound on a pasteur pipette tip, dried under vacuum and dissolved in 2 x SSC. RNA was removed by digestion with ribonuclease A at 100 µg/ml for 1 hour at 37°C. This was followed by phenol and chloroform extractions and ethanol precipitation as above. The DNA was dissolved in 10 mM Tris-HCl pH 7.5, 1 mM EDTA and used for restriction endonuclease digestion.

RNA Isolation

RNA was isolated from 50 x 150 cm^2 flasks. Nuclei were removed as described above. The post-nuclear supernatant was centrifuged at 16000 x g for 15 minutes to pellet the mitochondria. The post-mitochondrial supernatant was made 1% with respect to SDS and extracted with phenol and chloroform : isoamyl-alcohol (24:1) as described above. RNA was precipitated by the addition of 1/8 volume of 5 M NaCl and 2½ volumes of absolute ethanol. After precipitation at -20°C for 16 hours, the RNA was pelleted at 30 000 x g for 15 minutes, the pellet washed thrice in 70% ethanol, once in absolute ethanol, dried under vacuum and dissolved in sterile distilled water.

Restriction Enzyme Digestion and Transfer of Nucleic Acids to Nitrocellulose Paper

DNA was digested with the various restriction enzymes as suggested by the suppliers. In the case of HpaII and MspI digestions, enzyme was added at 2 units/µg DNA and incubated for 3 hours at 37°C. The digested DNAs were electrophoresed for 4 hours at 150 mA on 0.8% horizontal agarose gels in 40 mM Tris-acetate pH 7.8, 50 mM Na-acetate, 10 mM EDTA. Gels were stained with 0.1 mg/ml ethidium bromide and visualized under uv light. DNA was transferred to nitrocellulose paper as described by Southern (47). RNA was dotted onto nitrocellulose paper as described (50,51): briefly the nitrocellulose paper was soaked in sterile distilled water for 5 minutes, and 20 x SSC for 30 minutes. RNA was denatured at 65°C for 5 minutes, spotted on the nitrocellulose paper, dried under a lamp and baked at 80°C for 2 hours.

Nucleic Acid Hybridization

The filters were prehybridized for 1 hour at 68°C in 4 x SET (SET = 150 mM NaCl, 2 mM EDTA, 30 mM Tris-HCl pH 8.0), 10 x Denhardt [Denhardt = 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% BSA (48)], 0.1% pyrophosphate, 0.1% SDS and 50 µg/ml E. coli DNA. Hybridization was with either ^{32}P-labelled nick-translated DNA (49) or with ^{32}P-labelled cDNA (45) at 0.5 - 2 x 10^6 dpm/ml, in the above hybridization solution at 68°C for 20-24 hours. After hybridization, the filters were washed for 1 hour at 68°C in 4 x SET, 10 x Denhardt, 0.1% pyrophosphate and 0.1% SDS, followed by one wash at room

temperature in 1 x SSC and 0.5 x SSC for 1 hour each. The filters were exposed to Du Pont Cronex 4 X-ray film for 3-4 days.

Synthesis of Complementary DNA

cDNA was synthesized in 100 µl reaction volumes containing 150 mM NaCl, 50 mM Tris-HCl pH 8.2, 6 mM $MgCl_2$, 10 mM Dithiothreitol, 4 µg/ml oligo-$d(T)_{12-18}$, 1 mM each of dATP, dGTP, dTTP and 20 µM of ^{32}P-dCTP (400 Ci/mmole), 5 µg RNA and 75 units of AMV-reverse transcriptase. After incubation at 42°C for 2 hours, the reaction was stopped by the addition of EDTA to a final concentration of 50 mM, and passed over a Sephadex G-50 column (10 cm x 0.7 cm). ^{32}P-labelled cDNA was hybridized to southern blots of pCg45 and pCg54 as described for hybridization of genomic blots to nick translated DNA.

RESULTS

Collagen Synthesis in Normal and Transformed Fibroblasts

In order to quantitate the collagen produced by the two cell lines, cells were labelled with ^{3}H proline for 16 hours. An enriched collagen sample was prepared by precipitation with 5 M NaCl as described in Materials and Methods. Figure 1 shows the autoradiogram of the ^{3}H proline-labelled samples after electrophoresis on 5-11% gradient SDS-polyacrylamide gels. Two prominent bands, resistant to pepsin but sensitive to collagenase, could be seen in the case of normal fibroblasts, corresponding to α1(I) and α2 chains of type I collagen. In the samples derived from transformed fibroblasts, only one prominent band corresponding to an α1 chain was observed, with perhaps a trace of some protein in the α2 region. This major band was not α1(I), since its electrophoretic mobility was greater than that of the α1(I) chain produced by

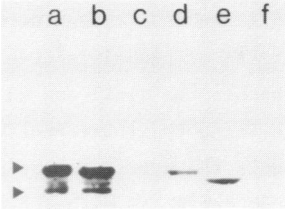

Figure 1. SDS polyacrylamide gel electrophoresis of collagens from WI-38 (lanes a - c) and from SVWI-38 (lanes d - f) fibroblasts. Cells were labelled with ^{3}H proline for 16 hours and 5 M NaCl precipitable proteins were analysed after treatment with pepsin (lanes a and d), treatment with pepsin and reduction with β-mercaptoethanol (lanes b and e), and digestion with collagenase (lanes c and f). Arrows indicate the position of migration of α1(I) and α2 chains.

normal fibroblasts. Analysis of the samples on SDS-urea gels (59), which separate the different α1 chain from each other, confirmed these results (data not shown). The autoradiograms were scanned densitometrically and the areas under the peaks were integrated; the transformed fibroblasts produced only 25-30% of the total collagen synthesized by normal fibroblasts. These results are in agreement with those of previous reports on decreased collagen production in SV40 transformed WI-38 fibroblasts (13), but the new finding is the complete shutdown of type I collagen synthesis with the continued synthesis of a minor collagen component. Since altered post-translational modification of the collagen chains in transformed cells could not be ruled out by these experiments, characterization of the mRNAs in the different cell lines was important.

Detection of Procollagen mRNA Sequences

The collagens are extremely conserved molecules and several studies have shown that cloned procollagen DNA sequences will cross hybridize between the same alpha chain clones of different species, but not between different alpha chain sequences within the same species, i.e. type I (α1) chicken procollagen cDNA will hybridize to type I (α1) mRNA or DNA from human or mouse, but not to type II or III α chains of chickens. Thus Meyers et al (52) and Weiss et al (53) have shown specific hybridization of chicken α1(I) and α2 procollagen cDNA clones (54,55) to human procollagen gene sequences, while others have shown specific hybridization of these chicken clones to both Drosophila (56,57) as well as mouse (58) procollagen gene sequences. Figure 2 shows hybridization of pCg 45 (α2 clone) and pCg 54 (α1 clone) to EcoR1 and Hind111 digested human and chicken DNAs. The patterns obtained for both human and chicken are very similar, suggesting no non-specific hybridization to non-collagen coding sequences. These results are similar to those of Myers et al (52). The chicken procollagen cDNA clones were therefore suitable for detecting human procollagen mRNA or genomic sequences.

In order to determine whether the α1(I) and α2 procollagen mRNAs were present in an untranslatable form, their presence was probed for using ^{32}P-labelled cDNA. Figure 3 shows the hybridization of ^{32}P-labelled cDNA from both WI-38 as well as SV WI-38 cells, to either pCg 45 or pCg 54. There was complete absence of hybridizable α1(I) and α2 procollagen cDNAs in the SV WI-38 fibroblasts, whereas cDNA from WI-38 fibroblasts hybridized to both pCg 45 as well as to pCg 54, indicating the presence of procollagen α1(I) and α2 mRNAs in normal fibroblasts. Since the cDNA was made from total cytoplasmic RNA using oligo d $(T)_{12-18}$ as primer, it is possible that the α1(I)

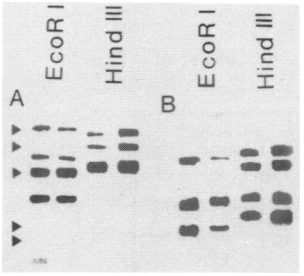

Figure 2. Comparison of EcoRl and Hindlll restriction endonuclease patterns of human (left lane of each pair) and chicken (right lane) procollagen gene sequences. DNAs were digested with the indicated restriction endonucleases, electrophoresed on 0.8% agarose gels and hybridized to either ^{32}P-nick-translated α1(I) {A} or α2{B} cDNA clones. Arrows indicate the position of migration of λHindlll markers.

and α2 procollagen mRNAs in the transformed cells were partially degraded, or that the poly (A) tails were absent. It is also possible that these mRNAs were present in such low quantities, that they were competed out by other mRNAs for the reverse transcriptase, or that they were not transported from the nucleus. In order to rule out these possibilities, total cellular RNA from WI-38 and SV WI-38 fibroblasts was immobilized onto nitrocellulose paper and hybridized to ^{32}P-labelled nick-translated pCg 45 or pCg 54. Thus even if the mRNAs were partially degraded or not efficiently reverse-transcribed into cDNA, they should still have been detected by this procedure. Table I shows the results obtained after hybridization of ^{32}P-labelled nick-translated clones to RNA dots of WI-38 and SV WI-38. Reticulocyte RNA was used as a control for background hybridization. These results clearly indicate the absence of α1(I) and α2 procollagen mRNA sequences.

Type I Procollagen Gene Restriction Pattern

Since no α1(I) and α2 procollagen mRNAs are synthesized in the transformed cells, some signal must exist for switching off these genes or some signal to "turn on" the genes is now absent. In order to determine whether large regions of the α1(I) and α2 procollagen genes were deleted, WI-38 and SV WI-38 fibroblast DNAs were digested with EcoRl, Hindlll, Kpnl, and Haelll. Figure

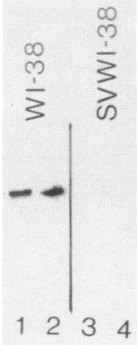

Figure 3. Detection of procollagen mRNA sequences in WI-38 and SVWI-38 fibroblasts. ^{32}P-labelled cDNA to WI-38 (lanes 1 and 2) and SVWI-38 (lanes 3 and 4) were hybridized to pCg 54 {α1(I)} and pCg 45 {α2} procollagen cDNA clones immobilized onto nitrocellulose paper. Lanes 1 and 3 indicate hybridization to pCg 54 and 2 and 4 to pCg 45.

Table 1. Detection of type I procollagen sequences in WI-38 and SVWI-38 fibroblasts. Total RNA was immobilized onto nitrocellulose paper as described in Materials and Methods, and hybridized to nick translated α1(I) and α2 procollagen cDNA clones.

Probe	μg RNA Immobilized	DPM ^{32}P Probe Hybridized		
		Reticulocyte	WI-38	SVWI-38
α1(I)	0.1	----	188	15
	1.0	----	760	21
	10.0	23	4020	44
	20.0	34	6500	89
α2	0.1	----	150	25
	1.0	----	890	18
	10.0	36	2700	50
	20.0	47	4410	90

4a and 4b show that the restriction patterns for both WI-38 and SV WI-38 were identical when restricted with the enzymes EcoRl, Hindlll, Kpnl, and Haelll, which do not have CpG dinucleotides in their recognition sequences and therefore would not be sensitive to CpG methylation. This applies both to the α1(I) as well as the α2 genes. However, Hpall and Mspl digests showed striking differences in the restriction pattern of both α1(I) and α2 genes (Figure 5). In the α1(I) gene of WI-38 fibroblasts, there were five Hpall fragments ranging in size from 0.3 to 1.8 kb. Mspl digestion showed that very few methylated sites were present in the α1(I) gene. The same applied to the α2 gene, with low molecular weight fragments ranging in size from 0.6-2.5 kb. However, in the transformed cells, these low molecular weight bands were absent in both Hpall and Mspl digested samples. These results indicate extensive hypermethylation of the procollagen α1(I) and α2 genes. The finding that Mspl fails to generate the 0.4-2.5 kb fragments can be explained by the fact that the enzyme, although insensitive to methylation of the internal cytosine and able to cleave at $C^{m}CGG$, fails to cut at $^{m}CCGG$ or at $^{m}C^{m}CGG$. The only explanation therefore is that the latter two methylated states were also present. In order to monitor the completeness of digestion, λDNA was added to WI-38 or SV WI-38 in parallel incubations. In these incubations the λDNA was completely digested, indicating the absence of an inhibitor of

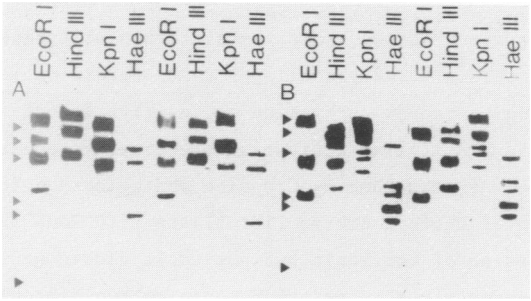

Figure 4. Restriction endonuclease digestion patterns of WI-38 (left 4 of each set) and SVWI-38 (right 4 of each set) procollagen gene sequences. DNAs were digested with the indicated restriction endonucleases and hybridized to either nick-translated pCg 54 (A) or pCg 45 (B) procollagen clones. Arrows indicate the position of migration of λHindlll markers.

the restriction enzymes or impure preparations of DNA. It is therefore clear that there were no large deletions in the α1(I) and α2 procollagen genes in the transformed fibroblasts, but that they may, in fact, be extensively methylated.

DISCUSSION

Previous experiments have shown that transformation of mouse embryo fibroblasts with mouse sarcoma virus results in altered collagen gene expression, in that the overall collagen synthesis is decreased to about 10% of the normal (10,14,15). There is also the activation of another minor collagen component

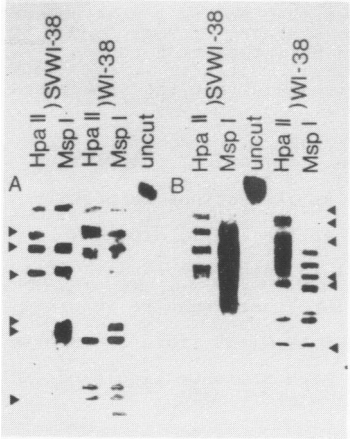

Figure 5. Detection of methylated sites within the procollagen genes of normal (WI-38) and SV40-transformed (SVWI-38) fibroblasts. DNAs were digested with either Hpall or Mspl and hybridized to pCg 54 (A) or pCg 45 (B). Arrows indicate the position of λHindlll markers.

(10,14,61). We have now shown that human diploid fibroblasts transformed with the DNA tumour virus (SV40) cease the synthesis of type I collagen. The mechanism by which this altered procollagen gene expression is achieved would not appear to be due to random activation or inactivation of stretches of DNA upon transformation. A similar activation of normally non-expressed genes upon transformation was described by Groudine and Weintraub (60), who found that transformation of chicken embryo fibroblasts with Rous Sarcoma virus results in the expression of embryonic but not adult globin genes. However, using the mouse sarcoma virus transformed mouse embryo fibroblasts, Parker and Fitschen (46) have shown that no globin genes are activated, although procollagen gene transcription is greatly reduced while a minor species of collagen which behaves similarly to type III α chains on carboxymethylcellulose is activated (61).

Exactly how this specific activation and depression of certain genes occurs is still uncertain. The only obvious difference between the pro α1(I) and pro α2 genes in the normal and SV40 transformed fibroblasts is the hypermethylation of these genes upon transformation. Similar results have been obtained in avian erythroblastosis virus-transformed cells (31). Normal chicken erythroblasts produce the adult β, α^A and α^D globins, but upon transformation with AEV, β and α^D globin gene transcription is shut off, whereas α^A genes continue to be transcribed. Further studies have shown that the inactivated β and α^D globin genes are hypermethylated whilst the α^A genes are unaffected by transformation. It is interesting that such similar findings of hypermethylation with inactivation of these two different genes in different systems should exist. It is therefore possible that hypermethylation may be a universal phenomenon associated with altered gene expression upon viral transformation, especially in view of the fact that AEV is a RNA tumour virus whilst SV40 is a DNA tumour virus. It would be interesting to see whether all DNA tumour viruses or all RNA tumour viruses affect the same genes, and in the same manner.

That the SV40 genome is not integrated with the α1(I) and α2 procollagen genes or that transformation does not result in gross alterations in procollagen gene structure is demonstrated by the fact that the EcoR1, HindIII, KpnI, and HaeIII restriction patterns are identical in both cell lines. It is also unlikely that viral transformation of WI-38 fibroblasts results in gross hypermethylation of genomic DNA since both WI-38 and SV WI-38 fibroblasts have the same 5-methylcytosine content as determined by high pressure liquid chromatography (42).

Several lines of evidence indicate that methylation of cytosines plays a role in gene expression at the transcriptional level (26-41). Additional evidence comes from the use of inhibitors of DNA methylation (64,65). Treatment of appropriate cells with such inhibitors (e.g. 5-azacytidine) causes the activation of normally dormant retrovirus genes (51,62,63). The normally inactive ev-1 retrovirus locus in chicken cells are hypermethylated and insensitive to DNAse-1. On treatment with 5-azacytidine, the ev-1 locus is sensitized to DNAse-1, becomes hypomethylated, and is expressed (51).

Methylation could be inovlved in the regulation of gene expression via interaction of the DNA with specific proteins. Examples of these could be the high mobility group proteins (HMG 14 and HMG 17), or the DNA binding proteins which appear to be synthesized in larger quantities in SV WI-38 cells than in WI-38 cells (66). It is obvious that the chromosomal proteins play a very important role in the regulation of gene expression (for review see Ref. 67), but these proteins would require some signal from the DNA in order to recognise genes or families of genes, and DNA methylation could be one such signal.

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