
Methylation of somatic vs germ cell DNAs analyzed by restriction endonuclease digestions

James Kaput and Thomas W.Sneider¹

Department of Biochemistry and Graduate Faculty of Cellular and Molecular Biology, Colorado State University, Fort Collins, CO 80523, USA

Received 6 August 1979

ABSTRACT

Bacterial restriction endonucleases containing the dinucleotide CpG in their cleavage sequences were used to compare the methylation patterns of primarily repeated DNA sequences in (1) bovine somatic cell native DNAs vs bovine sperm cell native DNA and (2) native vs renatured bovine liver and sperm cell DNAs. The restriction patterns of sperm native DNA differ markedly from those of somatic cell native DNAs when using Hpa II, Hha I, and Ava I but not when using the enzymes Eco RI and Msp I. Digestion patterns of germ cell renatured DNA differed significantly from those of germ cell native DNA when using Hpa II but not when using Msp I or Eco RI. The results may not be due to artifacts of renaturation of the DNAs. The results are consistent with the concept that germ cell DNA may be strand asymmetrically hemimethylated. The data also suggest that methylation of the 5'-cytosine in the sequence CCGG renders this site insensitive to cleavage by Msp I.

INTRODUCTION

DNAs isolated from germ cells of a wide variety of organisms are deficient in 5-methylcytosine in comparison to the 5-methylcytosine content of DNA from somatic cells of the same organism ². These findings, in conjunction with recent reports of restriction-like endonucleases in testes of African green monkey and mouse ³ and Xenopus laevis oocytes ⁴ led to the hypothesis that DNA methyltransferase may be absent or inactive in germ cells during the S-phase immediately preceding meiosis and that the resultant DNA duplicates may be strand asymmetrically hemimethylated ⁵.

In a number of cases Vanyushin et al.² found that germ cell DNAs had almost exactly 50% of the 5-methylcytosine content of corresponding somatic cell DNAs as would be predicted by this hypothesis. However, no information is available on either the nucleotide sequences methylated or the strand distribution of methylated bases in germ cell vs somatic cell DNAs. In the present experiments we use digestions of bovine somatic cell and germ cell DNAs with restriction endonucleases containing the dinucleotide CpG in their cleavage

site specificities to address these two questions.

METHODS

Novikoff rat hepatoma cells (line N1-S1) were grown in suspension culture as previously described ⁶. Bovine somatic cell DNAs were prepared from tissues of freshly slaughtered animals. Bovine semen was obtained from the Colorado State University Bull Farm.

DNAs were prepared as follows: frozen bull semen (1.8×10^9 sperm) was thawed, sperm harvested by centrifugation, washed twice with isotonic saline, and DNA isolated by the method of Borenfreund et al.⁷ except that all solutions contained 10 mM disodium EDTA and that nuclease-free Pronase and DNAase-free RNAase digestions were incorporated into the protocol. Bovine somatic cell DNAs were prepared from nuclei isolated from fresh tissues. Novikoff rat hepatoma cell DNA was made from isolated nuclei of logarithmically growing cells. DNAs were isolated from the nuclei using a previously described procedure ⁸ modified only in that all solutions were 10 mM in disodium EDTA. Hemimethylated Novikoff rat hepatoma cell DNA was obtained as follows: cell cultures (800 ml total; 500,00 cells/ml) were treated with the mitotic inhibitor Nocodazole (2.5×10^{-6} M) for 10 hours, harvested, washed once in growth medium (Swim's No.69C), resuspended in growth medium containing hydroxyurea (5×10^{-3} M) for 6 hours, harvested, washed once in methionine-free S69C, resuspended in S69C that was 10^{-6} M in methionine and 10^{-4} M in ethionine, and reincubated for one synchronous round of DNA synthesis (8 hours). The DNA was then isolated from purified nuclei as previously described ⁸. The putatively hemimethylated DNA produced by this method is ca. 70% nonmethylated in the newly made DNA strand ^{5,9,10}.

DNA renaturations: the relevant DNA was dissolved in 50% formamide-0.015 M NaCl-0.0015 M sodium citrate-10 mM disodium EDTA (pH 7) at concentrations ranging from 0.76 to 1.66 mg/ml, the samples made 0.75 M in NaCl by adding solid NaCl, denatured at 75°C for 30 minutes, and renatured at 37°C to C_0t 's ranging from 2600 to 4600. After renaturation the samples were dialyzed against two changes of 1000 volumes of 25 mM Tris Cl-10 mM EDTA (pH 7.4) for three hours and at 4°C using a microdialysis chamber.

Endonuclease digestions were performed using Eco RI, Hha I, and Hpa II from Bethesda Research Labs. and Msp I and Ava I from New England Biolabs. Digestions were carried out under conditions specified by the supplier except that concentrations of Tris buffer, EDTA, and Mg^{++} were adjusted as

necessary to correct for Tris buffer and EDTA present in the DNA substrate solutions. Endonuclease digestions were performed at 5 times the enzyme concentrations required to completely digest comparable amounts of λ DNA. Nevertheless, tests for completeness of digestion of the various DNAs were run with all assays *viz.* 1/50th of the digestion mixture (enzyme plus sample DNA) was removed at the start of the reaction, added to 2 to 4 μ g of λ DNA, and incubated for the same length of time as the digestions of sample DNAs. An aliquot of this digest of λ DNA was then analyzed on the same gel used for the digests of sample DNAs. This procedure scores for complete digestion of the λ DNA and, by inference, the completeness of digestion of the sample DNA. Additional experiments (which were not routinely run) used variation in the amount of restriction endonuclease used or in the length of the digestion to show that the results reported here are not due to partial digestions. [None of these data are shown here to conserve space but are available upon request.]

Agarose gel electrophoresis was performed using 20 x 30 x 0.5 cm slab gels varying from 1% to 2% agarose in standard electrophoresis buffer (0.04 M Tris base-0.005 M sodium acetate-0.001 M EDTA, pH 7.85). Enzyme digests were made 5% in glycerol-0.0005% in bromphenol blue prior to loading into the gel slots. Usually 25 μ l was loaded per slot; quantities of DNA loaded are noted in the Figure legends. Electrophoresis was performed at 25°C at a constant voltage of 200 V until the bromphenol blue dye migrated ca. 14 to 15 cm from the origin (usually 9.5 hr). Gels were stained with ethidium bromide in water (0.5 μ g/ml) and destained in distilled water. Stained gels were photographed using a short wavelength UV transilluminator and Polaroid Type 55 P/N film.

Thermal melting profiles were obtained with a Gilford-modified Beckman DU spectrophotometer equipped with a Gilford Thermoprogrammer. DNAs were melted in 0.015 M NaCl-0.0015 M sodium citrate (pH 7) at a rate of 1°C/min from 25°C to 85°C. Absorbances at 260 nm were corrected for thermal expansion of solvent at each point plotted in the Figure.

Isopycnic CsCl centrifugations were performed by dissolving solid CsCl in 0.015 M NaCl-0.0015 M sodium citrate-10 mM EDTA (pH 7) and adjusting to a density of 1.704 g/cm³; 10 to 40 μ g of DNA sample in buffer were added to 4.9 ml of the CsCl solution. Centrifugation was in a SW50.1 rotor for 48 hr at 25°C and 35,000 rpm. Gradients were analyzed with an ISCO 640 fractionator equipped with a ISCO UA5 monitor. Refractive indices of each 0.1 ml fraction were measured with an Abbe' 3L Bausch and Lomb refractometer main-

tained at 25°C.

S₁-nuclease digestions: ca. 100 µg of a given DNA was digested with S₁-nuclease (CalBiochem) in 0.027 M sodium acetate-45 mM NaCl-1.5 mM ZnCl₂ (pH 4.4) for 1 hr at 37°C. The mixture was then dialyzed at 4°C against 25 mM Tris-Cl-10 mM EDTA (pH 8).

RESULTS

Methylation patterns of bovine somatic vs germ cell DNAs: Type II restriction endonucleases cleave DNAs lacking symmetrically disposed methylated bases in both strands of DNA at endonuclease recognition/cleavage sites; fully- or hemi-methylated sites will not be cleaved¹¹. The restriction pattern of a DNA thus reflects the methylation status of bases in given cleavage sequences. Several bacterial restriction endonucleases that include CpG in their recognition sequences (much 5-methylcytosine in eukaryotic DNAs occurs in this dinucleotide¹²⁻¹⁴) have been used to delineate nucleotide sequence specificity of eukaryotic DNA methylation¹⁵⁻²⁰. We wished to determine if the sequence specificity of DNA methylation in bovine sperm DNA was altered since it contains just half the 5-methylcytosine content of bovine somatic cell DNAs. We digested native DNAs from bovine thymus, kidney, liver, and sperm with Eco RI, Msp I, Hpa II, Hha I, and Ava I and compared their DNA restriction fragment patterns (Figures 1 and 2).

Eco RI digestion (insensitive to methylated cytosines) yielded 5-6 major fragments of similar mobilities from all bovine DNAs. The restriction patterns of thymus, kidney, and liver DNAs were qualitatively similar to each other using Msp I, Hpa II, or Hha I. Some differences in fainter bands can be seen between digests of the somatic DNAs for given restriction endonuclease digestions. Such differences may reflect differences in the amounts of DNAs added to the digestion mixtures. Although every effort was made to digest and analyze equal amounts of the various DNAs, significant measuring error could have been introduced when taking microliter aliquots of very viscous DNA solutions. However, comparison of the digestion patterns of sperm native DNA vs somatic cell native DNAs shows clearcut differences using Hpa II, Hha I, and Ava I. These differences are most evident in the composite shown in Figure 2 above. Msp I digests of bovine somatic and germ cell DNAs are similar but at least three fragments present in digests of somatic DNA are missing or under-represented in sperm DNA (see arrows, Fig.1).

The discrete restriction fragments seen could arise from restriction of re-

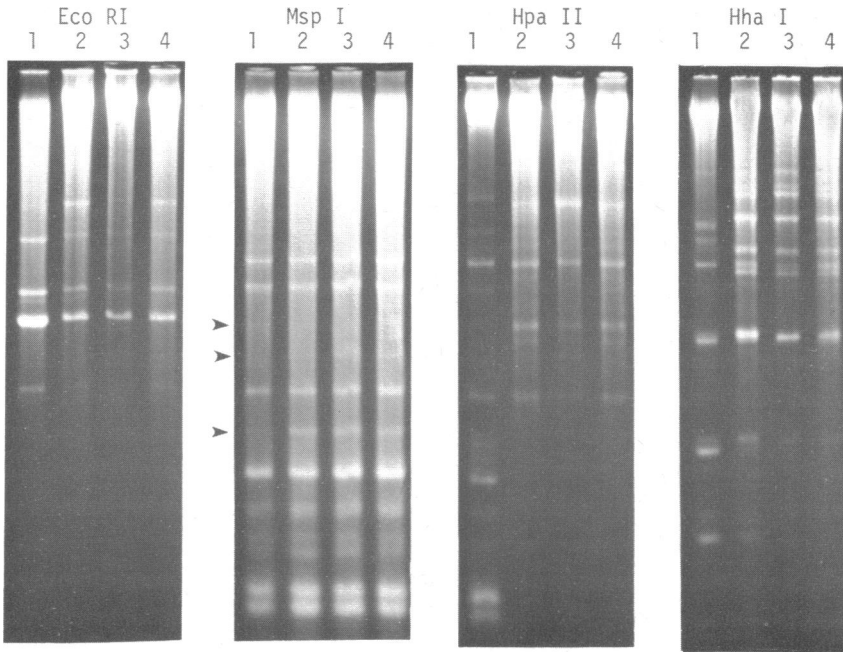


Figure 1. Eco RI, Msp I, Hpa II, and Hha I digests of bovine somatic native DNAs vs bovine sperm native DNA. Lanes 1,2,3, and 4 refer respectively to digests of sperm, liver, kidney, and thymus DNAs for each enzyme used. All digests run at 5 μ g of DNA per slot and on 2% agarose gels.

peated sequence DNAs and/or from restriction of a mitochondrial DNA contaminant. The possibility of contamination with mitochondrial DNA is low since somatic cell DNAs were isolated from Triton X100-purified nuclei and sperm cell DNA was isolated with a method that lyses mitochondria before chromatin in sperm heads is harvested. Moreover, the maximum total amount of mitochondrial DNA that could be present as contaminant in the 5 μ g of DNA analyzed would be 0.05 μ g which, in itself, is too low to be visualized on gels.

Since satellite DNAs often contain repeat sequence DNAs, the differences between digests of somatic and sperm cell DNAs might have arisen from different complements of satellites. Isopycnic centrifugations of native DNA preparations from liver and sperm yielded similar but not identical profiles (see Fig.6). Both DNAs had main band and satellite DNA peaks but the amounts of DNA in the various satellites (in proportion to the main band) differed between liver and sperm DNA. These differences, however, are artifacts that

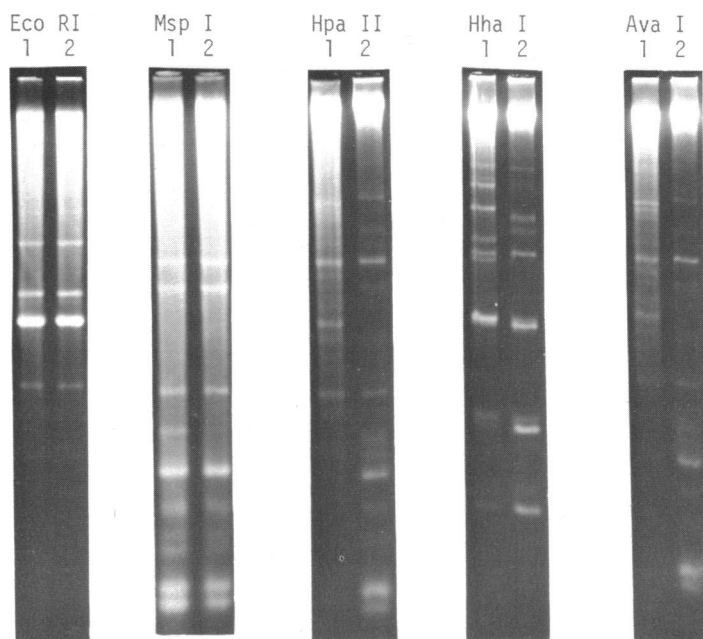


Figure 2. Comparison of Eco RI, Msp I, Hpa II, Hha I, and Ava I digests of bovine liver native DNA vs bovine sperm native DNA. Lanes 1 and 2 refer respectively to digests of liver and sperm DNAs for each enzyme used.

reflect a different degree of shearing of the two DNA preps viz. the CsCl profile of the less-sheared liver DNA can be converted to that seen with the more highly sheared sperm DNA by subjecting the liver DNA to shearing through a 26 gauge needle. Moreover, the similar Msp I digestion patterns between liver and sperm DNA suggest that the complements of repeated sequence DNAs between the liver and sperm DNA preps are similar.

These results therefore suggest that the distribution of methylated cytosines in sequences cleaved by these "CpG enzymes" are different in repeated sequence DNAs in bovine somatic vs germ cell DNAs. This result might arise from germ cell DNA methyltransferase(s) of differing sequence specificity(ies) than somatic cell DNA methyltransferase(s) or from the presence of the same sites but in a nonmethylated state in bovine germ cell DNA (see DISCUSSION).

Test for strand asymmetric hemimethylation of DNA: If germ cell DNA is synthesized in the premeiotic S-phase in the absence of DNA methyltransferase activity, the resultant duplexes would be asymmetrically hemimethylated. De-

naturation of that DNA followed by renaturation should yield reannealants of which 25% will be nonmethylated in both strands at given sites. Digestion of reannealed DNA with restriction endonucleases having CpG in their cleavage sequences should reveal some shift of the DNA into lower molecular weight species as compared to hemimethylated native DNA. To test this approach, originally devised by Bird ^{16,17}, we prepared fully methylated DNA and DNA known to hemimethylated (see METHODS) from Novikoff rat hepatoma cells and subjected these DNAs in either their native or renatured forms to digestion with various restriction enzymes. The results are shown in Figure 3. Eco RI digestion patterns of both native and renatured fully methylated and hemimethylated Novikoff cell DNAs are identical with respect to the major restriction fragments although a faint minor band seen in digests of the native DNA is absent from digests of the renatured DNAs. Msp I digestion patterns of native

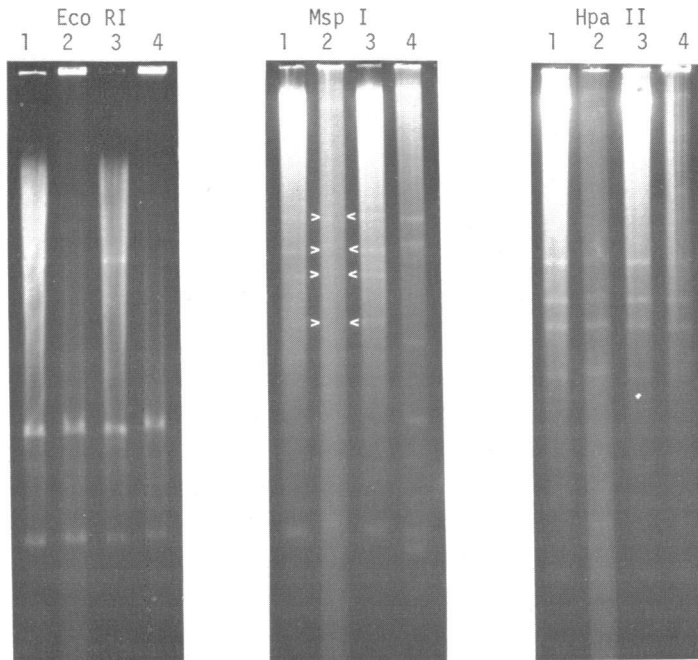


Figure 3. Eco RI, Msp I, and Hpa II digests of native vs renatured fully- or hemimethylated Novikoff cell DNAs. In each case lanes 1 and 2 show fully methylated DNA and lanes 3 and 4 show hemimethylated DNA. Lanes 1 and 3 show digests of native DNAs and lanes 2 and 4 show digests of renatured DNAs. Eco RI digests run on 1% gels; Msp I and Hpa II digests run on 2% gels. All native DNAs were loaded at 3 μ g per slot; renatured DNAs for Eco RI, Msp I, and Hpa II were loaded at 15 μ g, 9 μ g, and 20 μ g per slot respectively.

and renatured fully methylated and native hemimethylated DNAs are equivalent (white arrows on the Msp I digest of renatured fully methylated DNA denote bands equivalent to those seen in the native DNA digest but which do not reproduce well on the positive print) but the Msp I digest of renatured hemimethylated Novikoff cell DNA is significantly altered from the pattern for native hemimethylated DNA. Hpa II digestion patterns appear identical between native and renatured fully- or hemi-methylated Novikoff cell DNAs.

Several inferences can be drawn from the Hpa II and Msp I digestions of a Novikoff cell DNA which is known to be strand asymmetrically hemimethylated. Hpa II cleaves only if the internal cytosine in CCGG is not methylated ²¹. If Novikoff cell hemimethylated DNA contained $\begin{matrix} \text{CMGG} \\ \text{GGCC} \end{matrix}$, then 25% of the renaturants would be $\begin{matrix} \text{CCGG} \\ \text{GGCC} \end{matrix}$. The Hpa II pattern of hemimethylated renatured DNA would therefore differ from that of the native DNA. Figure 3 shows, however, that the Hpa II patterns of native and renatured Novikoff cell hemimethylated DNA were the same. This suggests that in the Novikoff cell repeated DNA sequences probed, the internal C in CCGG is not methylated.

Conversely, Msp I cleaves irrespective of methylation at the internal C in CCGG but it is not known if methylation of the 5'-cytosine in CCGG would protect against cleavage ¹⁹. If Novikoff cell hemimethylated DNA contained $\begin{matrix} \text{MCGG} \\ \text{GGCC} \end{matrix}$, then 25% of the renaturants would be $\begin{matrix} \text{CCGG} \\ \text{GGCC} \end{matrix}$. The Msp I pattern of the renatured DNA would then either (a) be the same as the pattern for native DNA provided that Msp I be insensitive to any methylated base in the CCGG sequence or (b) be different from the pattern for native DNA if Msp I is sensitive to methylation at the 5'-cytosine in CCGG. Figure 3 (cf. lanes 3 and 4) shows that the Msp I pattern of renatured hemimethylated DNA differs from the pattern for native hemimethylated DNA. This result suggests that in the Novikoff cell repeated sequence DNAs probed the 5'-cytosine in CCGG is methylated. Earlier analyses from this laboratory ¹⁴ on 5-methylcytosine localization in pyrimidine clusters of Novikoff cell DNA are consistent with this conclusion. The Msp I digestion results on a DNA known to be hemimethylated also suggest that the 5'-cytosine in CCGG may be the locus for methylation sensitivity of Msp I.

Asymmetric hemimethylation of bovine sperm DNA? The experiments above suggest that the approach of Bird ^{16,17} might be used to probe for asymmetric hemimethylation in repeated sequence fractions of complex mammalian DNAs. Application of this approach to native vs renatured bovine liver and sperm DNAs is shown in Figure 4. Eco RI digests of native vs renatured liver and sperm DNAs were all similar to each other (as expected since Eco RI is insensitive

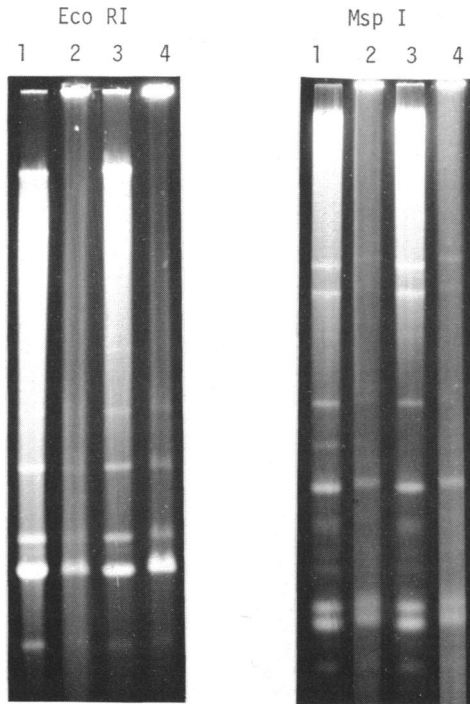


Figure 4. Eco RI and Msp I digestions of native vs renatured bovine liver and sperm DNAs. In each case lanes 1 and 2 show bovine liver DNA and lanes 3 and 4 show bovine sperm DNA. Lanes 1 and 3 show digests of native DNAs and lanes 2 and 4 show digests of renatured DNAs. Eco RI digest run on a 1% gel; Msp I digest run on a 2% gel. Native DNAs were loaded at 3 μ g per slot; renatured DNAs were loaded at 9 μ g per slot.

to methylated cytosine). This result also shows that the reannealed DNAs contain sufficient duplex character to regenerate a digestion pattern equivalent to that seen with native DNA. The Msp I digestion of native vs renatured liver and sperm DNAs also yielded similar patterns. This result could indicate that either bovine sperm DNA is not asymmetrically hemimethylated or that bovine sperm DNA is asymmetrically hemimethylated but Msp I cleavage sites are not DNA methylation sites in bovine DNA. The latter possibility gains credence from the results shown in Figure 5. Hpa II digestions of renatured sperm DNA show a markedly different pattern than for native sperm DNA. The photographic negatives of lanes 1 and 2 for the Hpa II digest (Fig.5) were scanned densitometrically and the two scans aligned using the peaks of the two lowest mo-

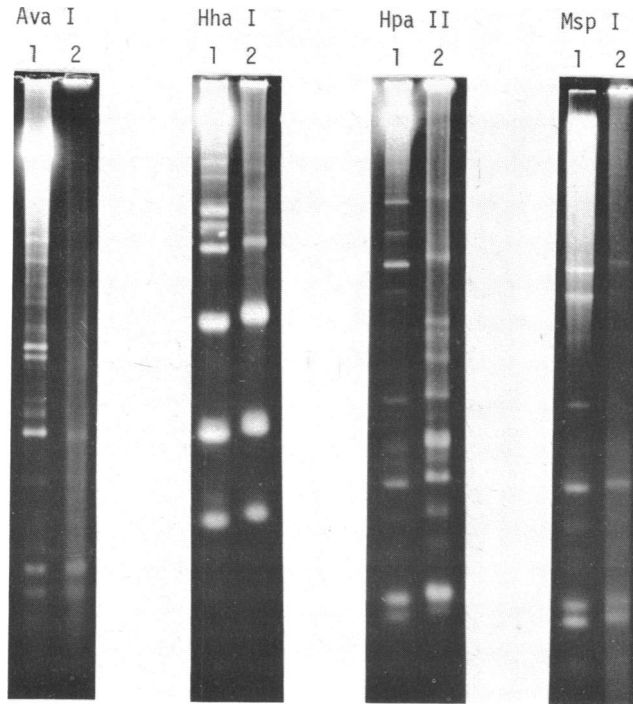


Figure 5. Ava I, Hha I, Hpa II, and Msp I digestions of native vs renatured bovine sperm DNA. In each case lane 1 shows the digestion of native DNA and lane 2 shows the digestion of renatured DNA. All digests run on 2% gels loaded with either 3 μ g of native DNA or 9 μ g of renatured DNA.

lecular weight fragments as reference points (Figure 6). Although the background is high, the scans do show the appearance of new fragments of different mobilities between digests of native vs renatured sperm DNA.

Some differences are also noted between native and renatured sperm DNA using Hha I (differences also observable densitometrically; data not shown). The background on the Ava I digest is too high to determine if this enzyme can also differentiate between native and renatured sperm DNA. Digests of bovine liver DNAs using Eco RI, Msp I, Hpa II, Hha I, and Ava I generally showed no differences between native vs renatured forms except that some higher molecular weight fragments present in digests of liver native DNA were absent in digests of liver renatured DNA. This difference is attributed to inadequate regeneration of longer stretches of duplex DNA in the renaturant from whence higher molecular weight restriction fragments might be generated

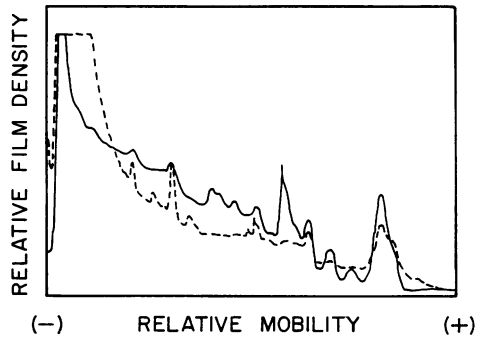


Figure 6. Densitometric analysis of electropherograms of Hpa II digests of bovine sperm native DNA (-----) and bovine sperm renatured DNA (——). Scans of photographic negatives of gels (see Fig.5) made with a Joyce-Loebl densitometer.

(see DISCUSSION).

Since the positive control (Fig.3) suggests that this general approach can detect strand asymmetric hemimethylation of DNA, the Hpa II (and possibly the Hha I) digestion provides some evidence for strand asymmetric hemimethylation of bovine germ cell DNA. The Hpa II digestion results also indicate that in bovines the internal cytosine in CCGG is methylated in the repeated sequence DNAs probed.

Conversely, the absence of differences in Msp I digestion patterns of native vs renatured sperm DNAs suggests that the 5'-cytosine in CCGG is not methylated. This conclusion is in accord with the fact that Hpa II -- but not Msp I -- digests of bovine somatic cell native DNA vs bovine sperm cell native DNA were markedly different. Precisely the opposite results with native vs renatured fully- and hemi-methylated Novikoff rat hepatoma cell DNAs may indicate that the sequence specificity of mammalian DNA methylation may be somewhat broader than has been proposed ^{22,23}.

To determine if renatured DNAs were enriched in special species of DNA that might generate the restriction patterns noted, the various reannealants were subjected to isopycnic CsCl centrifugation. The results are shown in Figure 7. The profiles for native DNAs from bovine liver and sperm are similar but not identical (commented upon earlier in this paper). Bovine liver renatured DNA shows some enrichment in G+C-rich species but sperm renatured DNA is not similarly enriched. The different CsCl gradient profiles for liver vs sperm renatured DNAs could be due to several factors. The bou-

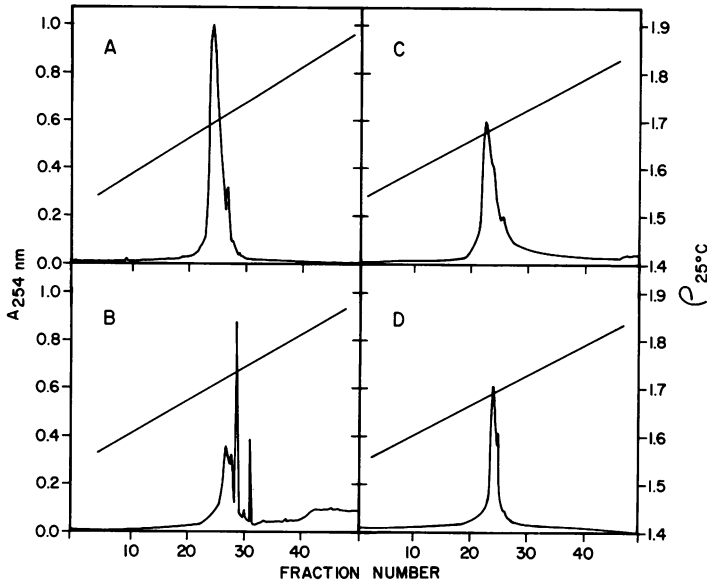


Figure 7. Neutral cesium chloride centrifugation profiles of native *vs* renatured bovine liver and sperm DNAs. (A) bovine liver native DNA, (B) bovine liver renatured DNA, (C) bovine sperm native DNA, (D) bovine sperm renatured DNA. In all cases about 35 μ g of DNA was analyzed.

yant densities of reannealants are higher than for native DNAs. This might be due to the presence of single-stranded regions in renatured DNA since, upon treatment of the renaturants with S η -nuclease, the bouyant densities of the renaturants become very similar to those of the corresponding native DNAs. A factor related to this is that the liver DNA preparation is less sheared than the DNA preparation from sperm (as deduced from agarose gel electrophoreses of native DNAs from liver and sperm; data not shown). Since renaturation of DNA is sensitive to the lengths of the reactant DNA molecules, one might expect a population of reannealants biased toward repeated sequence DNAs in a less sheared DNA preparation. However, the crucial comparison is between native *vs* renatured sperm DNA or between native *vs* renatured liver DNA. Enrichment of reannealants in repeated sequence DNAs has the effect of increasing the sensitivity of the restriction analyses since the discrete fragments seen in the digests are probably generated from repeated sequence DNAs.

Thermal melting studies indicate the duplex nature of the reannealants

(Figure 8 and Table 1) and the fact that the G+C contents thereof are not markedly enhanced (G+C contents calculated from T_m are lower than would be predicted from the bouyant density data due to the breadth of the hyperchromic shift as well as the artifactually higher bouyant density values caused by single-strandedness in the renatured DNAs). The effect of single-strandedness in reannealants on restriction enzyme digestions was examined by treatment of renatured DNAs with S_1 -nuclease prior to restriction endonuclease digestion. One example of this study is shown in Figure 9. Although S_1 -nuclease treatment per se produces a broad size range of native and renatured DNAs, the Eco RI digests of native and renatured fully methylated and hemi-methylated Novikoff cell DNAs produced similar major bands regardless of whether or not the DNAs were pretreated with S_1 -nuclease. The same result was obtained using native and renatured bovine sperm DNA and the enzyme Hha I.

The data therefore suggest that the renatured DNAs used contain significant amounts and lengths of duplex DNA (probably repeated sequence DNAs) cleavable by the restriction endonucleases used as probes. Additionally, new discrete fragments in restriction digests of reannealed sperm DNA might

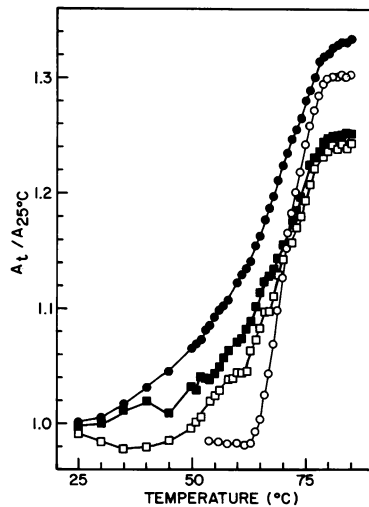


Figure 8. Thermal melting profiles of native vs renatured bovine sperm DNA with or without treatment with S_1 -nuclease. (\bullet , \circ) refers to sperm native DNA treated (\bullet) or not treated (\circ) with S_1 -nuclease. (\blacksquare , \square) refers to sperm renatured DNA treated (\blacksquare) or not treated (\square) with S_1 -nuclease. In all cases the initial absorbance at 260 nm was adjusted to 0.2 prior to beginning the melting analyses.

TABLE 1
T_m AND G+C CONTENT OF NATIVE AND RENATURED DNAs

DNA	STATE	S ₁ NUCLEASE	T _m	% HYPERCHROMICITY ^(b)	% RESISTANT TO ^(c) S ₁ NUCLEASE	% G+C FROM T _m
Novikoff cell +/+ ^(a)	Native	-	70.5	27.5	nd ^(d)	40.5
	Renatured	-	65.3	10.5	100.	27.8
	Renatured	+	69.1	19.6	10.2	37.1
Novikoff cell +/-	Native	-	70.6	37.9	nd	40.7
	Renatured	-	68.0	22.0	100.	34.4
	Renatured	+	69.2	17.6	28.8	37.3
Bovine liver	Native	-	70.1	32.1	nd	39.5
	Native	+	72.2	31.6	nd	44.5
	Renatured	-	67.3	23.2	100.	32.6
	Renatured	+	70.5	28.4	11.0	40.6
Bovine sperm	Native	-	70.7	29.5	nd	40.8
	Native	+	68.7	32.9	nd	36.2
	Renatured	-	65.9	26.1	100.	29.3
	Renatured	+	69.2	33.7	17.1	37.3

(a) The +/+ refers to fully methylated and the +/- refers to artificially produced hemimethylated Novikoff rat hepatoma cell DNA.

(b) % hyperchromicity = $A_{260 \text{ max}} - A_{260 \text{ at } 25^{\circ}} / A_{260 \text{ at } 25^{\circ}} \times 100$.

(c) Determined by chemical analyses of trichloroacetic acid precipitable material after S₁-nuclease digestion.

(d) nd = not determined

not be expected as renaturation artifacts given the random nature of that process. Hence, the different restriction patterns noted between native vs renatured bovine sperm cell DNAs may not be due to renaturation artifacts.

DISCUSSION

Methylation patterns of repeated sequence DNAs in bovine somatic cell vs bovine germ cell DNAs: The data presented indicate different methylation patterns of repeated sequence DNAs in bovine germ cell native DNA compared to bovine somatic cell native DNAs. While this communication was in preparation, Waalwijk and Flavell reported differential methylation of the β globin gene in rabbit sperm DNA vs the β globin gene in various rabbit somatic cell DNAs²⁰. Interestingly, Drahovsky et al. just reported that inverted repetitive DNA sequences in mastocytoma cells (in cell culture) were hypermethylated compared to bulk DNA²⁴. The relationship of their findings to the present results remains to be explored.

A facile explanation for the present results is that bovine germ cells contain DNA methyltransferase(s) with different sequence specificity(ies) than the enzyme in bovine somatic cells. This explanation can not presently be ruled out but the results of the Msp I and Hpa II digestions decrease its

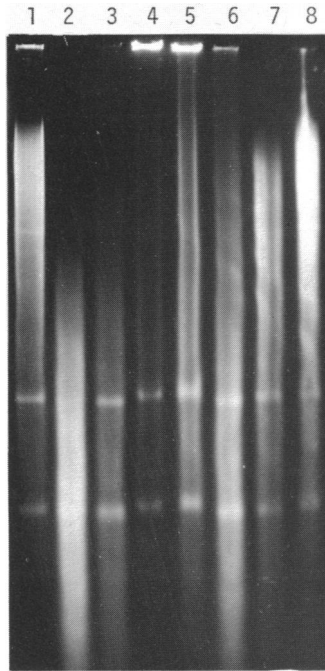


Figure 9. Eco RI digests of native vs renatured Novikoff rat hepatoma cell DNAs after pretreatment with S₁-nuclease. Lanes 1-4, digests of fully methylated DNA; lanes 5-8, digests of hemimethylated DNA. Lane 1, native DNA; lane 2, native DNA pretreated with S₁; lane 3, renatured DNA pretreated with S₁; lane 4, renatured DNA; lane 5, renatured DNA; lane 6, renatured DNA pretreated with S₁; lane 7, native DNA pretreated with S₁; lane 8, native DNA. Digests analyzed on a 1% agarose gel. All native DNAs loaded at 3 μg per slot; all renatured DNAs loaded at 9 μg per slot.

likelihood. Specifically, Msp I digests of native and renatured bovine somatic and germ cell DNAs were very similar if not identical (see Figs. 1 and 4) which implies that methylation at the 5'-cytosine in the sequence CCGG is negligible in the repeated sequence fractions of these DNAs. This implication is also supported by earlier dinucleotide¹² and pyrimidine cluster analyses¹³ of 5-methylcytosine in bovine DNAs. However, the internal cytosine in CCGG must be methylated in bovine somatic cell DNA since Hpa II digests thereof differ from Msp I digests of somatic native DNA. If the internal cytosine in CCGG had not been methylated, then the Hpa II and Msp I patterns would have been identical since both enzymes cleave at CCGG and there may not be significant methylation at the 5'-cytosine in CCGG of the repeated

sequence DNAs probed. The Hpa II sites in bovine sperm DNA must also be methylated at the internal cytosine since Hpa II digestion of renatured sperm DNA differed markedly from Hpa II digestion of native sperm DNA. Thus, both bovine somatic and germ cell DNA methyltransferases have specificity for the internal C in CCGG. This does not rule out, of course, the possibility that other DNA methylases with site specificities for different sequences are present or absent in germ vs somatic cells,

Another possibility for the different methylation patterns between somatic cell native DNAs and germ cell native DNA follows from the hypothesis mentioned in the INTRODUCTION and discussed in detail elsewhere ⁵. If the non-methylated strand of asymmetrically hemimethylated germ cell DNA were involved in a single strand crossover event in meiosis and were subsequently used as template for mismatch repair, the DNA made in the repair process may also then lack methylated cytosines since the hypothesis posits the absence or inactivity of DNA methyltransferases in meiosis. Hence, after crossover and repair synthesis, native germ cell DNA could then contain totally non-methylated sites susceptible to cleavage by the CpG-specific restriction endonuclease probes. This admittedly speculative explanation depends upon the validity of the hypothetical model ⁵. Inasmuch as that model receives some support from the present finding of strand asymmetric hemimethylation of bovine sperm DNA, this possibility should be given some consideration.

Strand asymmetric hemimethylation of sperm DNA: Bird employed restriction endonuclease digestion of native vs renatured *Xenopus laevis* ribosomal DNAs to probe for asymmetric hemimethylation. The present application of this approach to native vs renatured fully- and hemi-methylated DNAs from Novikoff rat hepatoma cells demonstrates its utility for repeated sequence fractions in complex bulk mammalian DNAs. It should be noted, however, that the higher molecular weight fragments apparent in digests of native Novikoff cell DNAs (both fully- and hemi-methylated) are faint or missing in digests of both corresponding renatured DNAs. This is contrary to the expected result i.e. regeneration of all fragments in renatured fully- and hemi-methylated DNAs although in the latter case the intensity of some bands should be decreased 25% due to generation of totally nonmethylated duplex sites and cleavage to new lower molecular weight restriction fragments. This discrepant result might be explained if the renatured DNA only contained lengths of duplex reannealant which, on the average, were less than the lengths of the higher molecular weight fragments seen in digests of native DNAs. Treatment of renatured fully- or hemi-methylated Novikoff cell DNAs with S₁-nuclease

followed by agarose gel electrophoresis does indicate that the average length of reannealed duplex is less than that for native DNA. In any event, pre-treatment of reannealants with S₁-nuclease does not alter subsequent restriction patterns -- a result which might have been predicted given the requirement for double-stranded DNA for restriction endonucleases. It should be emphasized, however, that the restriction endonuclease analyses as used in this study probe repeated sequence DNAs. Electropherograms of digests of renatured DNAs (analyzed by usually loading three times as much renaturant onto the gel as its native DNA counterpart) show that much of the DNA remains in the gel loading well and does not enter the gel. Hence, only discrete bands cleaved from duplex reannealants (11-17% of the total DNA in the reannealing mixtures of liver and sperm DNAs; see Table 1) are scored. We are attempting to isolate one or more discrete fragments from a non-CpG-specific restriction endonuclease digest of bovine sperm native DNA. Denaturation and reannealing of this discrete fragment followed by Hpa II digestion may circumvent problems associated with digestion of bulk renatured DNAs.

Eco RI digestions of native vs renatured bovine liver and sperm DNAs showed that a restriction endonuclease insensitive to methylated cytosines does not differentiate between either liver native DNA vs sperm native DNA or between native vs renatured liver or sperm DNAs. Hpa II digestion, however, clearly distinguishes renatured sperm DNA from native sperm DNA or from native or renatured liver DNA. Hha I digestions show some differences between native vs renatured sperm DNA but the differences are not as pronounced. Given the validity of this approach as shown by the digestion of native vs renatured Novikoff hepatoma cell DNA known to be hemimethylated, these results provide some evidence that bovine sperm cell DNA may be strand asymmetrically hemimethylated.

Methylation sequence specificity: The very similar Msp I digestion patterns of native vs renatured bovine somatic cell and germ cell DNAs (Fig.4) are, on their face, contrary to the conclusion just made in the preceding paragraph. However, the Msp I digestion results may be due to quite different methylation patterns in bovine DNA as compared to Novikoff rat hepatoma DNA and to the methylation site specificities that render DNA resistant to cleavage by Msp I and Hpa II. Msp I digests of Novikoff cell DNA known to be strand asymmetrically hemimethylated do show pronounced differences between the native vs renatured forms whereas the Hpa II digests do not exhibit these differences. These results imply that the internal cytosine in CCGG is not methylated in Novikoff cell repeated sequence DNAs since methyl-

ation at that site would protect against Hpa II cleavage and lead to different native vs renatured restriction patterns ²¹. The results also suggest that, since Msp I is insensitive to methylation of the internal cytosine in CCGG ¹⁹, the Novikoff cell repeated sequence DNA is methylated at the 5'-cytosine and that Msp I is sensitive to methylation at this site.

The Msp I and Hpa II digestions of native vs renatured bovine sperm DNA yielded precisely the opposite results i.e. Hpa II -- but not Msp I -- digestions differentiated between native vs renatured sperm DNA. Using the same reasoning as above, these results suggest that bovine repeated sequence DNAs are methylated at the internal cytosine in CCGG and not at the 5'-cytosine. The conclusion regarding the sequence specificity of Novikoff rat hepatoma cell DNA methylation is supported by our earlier analyses of the localization of 5-methylcytosine within pyrimidine clusters of Novikoff cell DNA ¹⁴. The conclusion regarding sequence specificity of bovine DNA methylation is supported by dinucleotide and pyrimidine cluster analyses performed in other laboratories ^{12,13}. Additionally, we have already reported that a highly purified DNA methyltransferase preparation from Novikoff rat hepatoma cells transmethylates native hemimethylated Novikoff cell DNA in vitro very efficiently but is minimally active using native putatively hemimethylated bovine sperm DNA as a substrate ⁵. We are currently purifying bovine DNA methyltransferase in order to perform the reverse specificity experiment. In sum, the current proposition that DNA methylation site sequence specificity is strongly conserved in mammalian species ^{23,24} deserves further examination in view of the present results.

The data raise a further quite interesting question. If germ cell DNA remains strand asymmetrically hemimethylated in the mature gamete, then the fate of that DNA is of interest. Restoration of DNA methylase activity in the fertilized egg prior to the onset of the first round of mitotic DNA synthesis would restore the methylation pattern of maternal and paternal chromatids. However, if the first round of DNA synthesis begins or is completed prior to restoration of DNA methylase activity, the methylation pattern could be disrupted i.e. the hemimethylated DNA would yield hemimethylated and totally nonmethylated daughter duplices. The hemimethylated daughter duplex could have its methylation pattern restored but the totally nonmethylated daughter duplex might be stable as such since DNA methylases apparently efficiently transmethylate only half-methylated sites ²⁵. The consequences of such a disruption of methylation pattern are purely speculative. But, if eukaryotic DNA

methylation is in fact tied to cognate restriction endonucleases (see^{3,4}) with the restriction enzymes having the same sequence specificities as the DNA methylases, then the presence of methylatable yet totally nonmethylated sites in somatic-type cell DNA may be of wide interest in terms of DNA re-arrangement phenomena as well as in regard to previous postulates concerning X-chromosome inactivation²⁶ and cellular differentiation²⁷.

ACKNOWLEDGEMENTS

This work was supported by grants (to TWS) from NIH-National Cancer Institute (CA-18719), the Research Corp. of America (No.7807), and a NIH-Biomedical Research Support Grant to Colorado State University. We thank the Interstate Packing Co. of Ft. Collins, CO and the Montfort Packing Co. of Greeley, CO for gifts of bovine tissues.

REFERENCES

1. To whom all correspondence should be addressed.
2. Vanyushin, B.F., Tkachevs, S.G., and Belozersky, A.N. (1970) *Nature* 225:948-949.
3. Brown, F.L., Musich, P.R., and Maio, J.J. (1978) *Nucleic Acids Res.* 5:1093-1106.
4. Benbow, R.M., Krauss, M.R., and Reeder, R.H. (1978) *Cell* 13:307-318.
5. Sneider, T.W., Kaput, J., Neiman, D., and Westmoreland, B. (1979) in Transmethylations, eds. E.Usdin et al. (Elsevier North Holland, N.Y.) pp.473-481.
6. Sneider, T.W. and Potter, V.R. (1969) *J.Mol.Biol.* 42:271-284.
7. Borenfreund, E., Fitt, E., and Bendich, A. (1961) *Nature* 191:1375-1377.
8. Sneider, T.W. (1971) *J.Biol.Chem.* 246:4774-4783.
9. Sneider, T.W., Teague, W.M., and Rogachevsky, L.M. (1975) *Nucleic Acids Res.* 2:1685-1700.
10. Kaput, J. and Sneider, T.W. (1978) *Fed.Proc.* 37:1737.
11. Arber, W. (1974) *Prog.Nucl.Acid Res.Mol.Biol.* 14:1-37.
12. Sinsheimer, R.L. (1954) *J.Biol.Chem.* 208:445-459.
13. Doskocil, J. and Sorm, F. (1962) *Biochim.Biophys.Acta* 55:953-959.
14. Sneider, T.W. (1972) *J.Biol.Chem.* 247:2872-2875.
15. Roy, P.H. and Weissbach, A. (1975) *Nucleic Acids Res.* 2:1669-1684.
16. Bird, A.P. and Southern, E.M. (1978) *J.Mol.Biol.* 118:27-47.
17. Bird, A.P. (1978) *J.Mol.Biol.* 118:49-60.
18. Singer, J., Roberts-Ems, J., and Riggs, A.D. (1979) *Science* 203:1019-1021.
19. Waalwijk, C. and Flavell, R.A. (1978) *Nucleic Acids Res.* 5:3231-3236.
20. Waalwijk, C. and Flavell, R.A. (1978) *Nucleic Acids Res.* 5:4631-4641.
21. Mann, M.B. and Smith, H.O. (1977) *Nucleic Acids Res.* 4:4211-4221.
22. Browne, M.J., Turnbull, J.F., McKay, E.L., Adams, R.L.P., and Burdon, R.H. (1977) *Nucleic Acids Res.* 4:1039-1045.
23. Browne, M.J. and Burdon, R.H. (1977) *Nucleic Acids Res.* 4:1025-1037.
24. Drahovsky, D., Boehm, T.L.J., and Kreis, W. (1979) *Biochim.Biophys. Acta* 563:28-35.

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25. Smith, H.O. (1979) *Science* 205:455-462.
26. Riggs, A.D. (1975) *Cytogenet.Cell Genet.* 14:9-25.
27. Holliday, R. and Pugh, J.E. (1975) *Science* 187:226-232.