Studies on the Influence of Cytosine Methylation on DNA Recombination and End-joining in Mammalian Cells*

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To test the influence of cytosine methylation on homologous recombination and the rejoining of DNA double strand breaks in mammalian cells, we developed a sensitive and quantitative assay system using extrachromosomal substrates. First, methylation was introduced into substrates in vitro with the prokaryotic SssI methylase, which specifically methylates the C-5 position of cytosine bases within CpG dinucleotides, mimicking the mammalian DNA methyltransferase. Next, methylated substrates were incubated in mammalian cells for a sufficient length of time to recombine or rejoin prior to substrate recovery. Results from bacterial transformation of the substrates and from direct Southern analysis demonstrate that cytosine methylation has no detectable effect on either DNA end-joining or homologous recombination. Thus, the components of the protein machinery involved in these complex processes are unaffected by the major DNA modification in mammalian cells. These results leave open the possibility that methylation may modulate the accessibility of these components to chromosomal DNA by altering local chromatin structure.

Two key processes in the maintenance of genomic integrity in mammalian cells are DNA end-joining, a nonhomologous process in which DNA breaks are rejoined, and homologous recombination. Both of these processes are mobilized in the repair of double strand breaks in chromosomal DNA (1) and in transfected extrachromosomal DNA (reviewed in Refs. 2 and 3). Homologous recombination of transfected DNA substrates is postulated to occur primarily by the nonconservative single strand annealing pathway (4, 5). In this pathway, the two substrates contain double strand breaks at or near their homologous regions on which an exonuclease (or helicase) acts to produce single strands. The homologous single strands anneal and subsequent processing steps result in the completion of the recombination event.

DNA end-joining has also been extensively studied by transfection of substrates into mammalian cells (2). Surprisingly, many different combinations of DNA ends can be rejoined efficiently in vivo. In addition to the precise ligation of compatible ends, noncompatible ends are also rejoined. For example, blunt-ended DNA ends can be rejoined to either 3' or 5' overhangs. The rejoining of the noncompatible ends frequently occurs within very short homologies near the DNA ends (2). Mechanistically, this type of end-joining may be similar to the single strand annealing pathway of homologous recombination, the key distinction being the length of the homology. Although the protein machinery involved in either recombination or DNA end-joining is not well characterized, a number of components, including exonuclease, DNA polymerase, ligase, and strand annealing proteins, can be expected to participate.

The major base modification of mammalian DNA, cytosine methylation, occurs on the C-5 position of cytosines within the context of CpG dinucleotides (reviewed in Ref. 6). Cytosine methylation has several demonstrated biophysical consequences on DNA and, thus, it may modulate the enzymatic activity of some of the components involved in recombination or end-joining. For example, methylation increases the melting temperature of naked DNA (7). It also has other effects, such as influencing the expression of DNA cruciforms (8), as well as the transition of DNA from the B form to Z form (9). However, it appears to have almost no influence on the intrinsic flexibility of DNA (10).

Functionally, methylation has a critical role in transcriptional regulation, and it has been implicated in both the establishment and maintenance of X chromosome inactivation and genomic imprinting patterns (6). The consequences of cytosine methylation in gene regulation may be the result of directly or indirectly altering the binding or activity of transcription factors and chromatin proteins (11). Although Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila have no detectable methylation, the importance of methylation in mammals has been underscored by gene knockout experiments in which it was found that mice defective in the DNA methyltransferase die during embryonic development (12). Methylation may also play a role in regulating the timing of replication. For example, the inactive X chromosome is replicated later than the active X chromosome in female cells (13, 14).

At present, it is unknown whether methylation affects homologous recombination in either mitotic or meiotic mammalian cells. However, it has been observed that recombination rates differ at identical chromosomal regions between males and females. Overall, females have a higher rate of recombination than males, although some chromosomal regions recombine more frequently in males than in females (15). Methylation patterns also differ between male and female gametes, with spermatogenic cells having an overall higher level of methylation than oogonic cells (16, 17). Thus, methylation could potentially directly or indirectly suppress recombination rates. Related to this, it has been demonstrated that methylation decreases the site-specific recombination of antigen recog-
tor genes, at least in some contexts. In this report, we have begun to address whether methylation has a direct affect on either homologous recombination or DNA end-joining in mammalian cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructions and Methylation Reactions—DNA manipulations were performed according to standard procedures (18). The Tn5 neo gene fragments were derived from plasmid pSV2neoM7. Plasmid pSV2neoM7 has a hybrid neo gene in which the 5' portion of the gene is derived from pSV2neo (19) and the 3' portion of the gene is derived from pMC1neoAP2 (20). The fragments that gave rise to pSV2neoM7 are the 961-bp Nde/Eagl fragment from pSV2neo, the 816 bp Eagl/BamHI fragment from pMC1neoAP2, and PstI/BamHI cleaved pUC19. The PstI and NdeI sites were blunted prior to ligation by treatment with T4 DNA polymerase and Klenow fragment, respectively. Plasmid Mneo was constructed by cloning the 1206-bp HindIII/BamHI Tn5 neo gene fragment from pSV2neoM7 into HindIII/BamHI-cleaved pUC19. The recombinant substrate M5neo was constructed by inserting the 885-bp HindIII/SphI Tn5 neo gene fragment of pSV2neoM7 into HindIII/SphI-digested pUC19. Plasmid M3neo was generated by ligating the 673-bp PstI/BamHI neo gene fragment of pSV2neoM7 into PstI/BamHI-digested pUC19. The latter two plasmids have 352 bp of homology between the PstI and SphI sites of the neo gene. Prior to transfection, M5neo was cleaved with Sphi, and M3neo was cleaved with PstI and AatII. Cleaved DNA was methylated in vitro with the SssI methylase according to the manufacturer’s instructions (New England Biolabs). The samples of unmethylated DNAs were incubated in methylation buffer without the SssI methylase, as a control.

Bacterial Transformation—Escherichia coli DH125, genotype araD139 Δ(ara, leu)7697 ΔlacX74 galU galK mcrA Δ(mrr-hsdRMS-mcrBC) rpsL deQ8801α-cZAM15 nupG recA1/F prsA1 lac+ idn- ZAM15, was prepared for transformation via electroporation as recommended by the manufacturer (Life Technologies, Inc.). Bacteria (40 μl) were mixed with 0.5 μl of DNA and electroporated in a 1.0-cm cuvette using a Bio-Rad gene pulser with a setting of 1.8 kV. They were then placed in 1 ml of SOC medium for a 1-h incubation at 37 °C prior to plating on ampicillin- or kanamycin-containing plates.

Cell Culture and DNA Recovery—COS1 cells were cultured in 150-cm² tissue culture flasks and transfected via electroporation. Cells were harvested at subconfluence and resuspended in phosphate-buffered saline at a concentration of 10⁶ cells/ml. A volume of 0.8 ml of cells was placed in a 0.4-cm cuvette and electroporated using a Bio-Rad gene pulser with a setting of 250 V, 960 μF. J ust prior to electroporation, 30 μg of each neo plasmid DNA was mixed with the cells. Cells were washed twice in phosphate-buffered saline immediately after electroporation to remove untransfected DNA and then incubated in tissue culture medium at 37 °C. To recover transfected DNA, cells were harvested after 4-h incubation. They were trypsinized and then washed three times in phosphate-buffered saline to remove any remaining untransfected DNA. Transfected DNA was recovered as described previously (21) and resuspended in 50 μl of H₂O. Southern analysis was performed using 1–2 μl of DNA according to standard procedures (18).

RESULTS

A Sensitive Assay for Extrachromosomal Homologous Recombination—To determine whether cytosine methylation influences the efficiency of extrachromosomal homologous recombination in mammalian cells, we developed a sensitive and quantitative assay based on bacterial transformation. Similar assay systems have been utilized previously (3). This assay also allows us to determine if methylation affects the DNA end-joining. The assay is sensitive and quantitative, since a large sample pool of plasmid DNA can be tested by its ability to confer antibiotic resistance to bacteria. Even more, individual recombination and end-joining products can be analyzed by recovering plasmid DNA from bacterial colonies, thus providing insights into repair processes at the molecular level.

Recombination substrates were constructed based on the parental Mneo plasmid. Mneo has an intact Tn5 neo gene and confers kanamycin resistance (Kan³) to bacteria (Fig. 1). The

\[ \text{Mneo} 3.9 \text{ Kb} \]

\[ \text{M5neo} 3.6 \text{ Kb} \]

\[ \text{M3neo} 3.3 \text{ Kb} \]

Fig. 1. Structure of the recombination substrates. The parental plasmid Mneo contains a functional Tn5neo gene, whereas the recombination substrates M5neo and M3neo contain 3'- and 5'-truncated neo genes, respectively. Base pair positions of relevant restriction sites are indicated for Mneo.

Tn5 neo gene expresses only in bacteria, so as to bypass any potential transcriptional effects on recombination and end-joining in mammalian cells. This plasmid and its derivatives do not contain a mammalian replication origin. The two intermolecular recombination substrates, M5neo and M3neo, contain 3'- and 5'-truncated Tn5 neo genes, respectively, and have an overlap of 352 bp of homologous sequences within the neo gene. Recombination within this homology restores a functional neo gene. To stimulate recombination, the plasmids are cleaved at the end of the homology region prior to transfection. M5neo is cleaved with Sphi (M5neoS), and M3neo is cleaved with PstI and AatII (M3neoAP). Restriction with each of these enzymes produces 4-base 3' overhangs, although the sequence of the overhang differs for each.

The experimental design is shown in Fig. 2. M5neoS and M3neoAP are methylated in vitro with a cytosine methylase and then electroporated into mammalian cells. Cells are incubated for 4 h, after which time plasmid DNA is recovered. We chose 4 h, since previous results have shown that recovery of recombined DNA is optimal at this time point. The recovered DNA is electroporated into E. coli, and colonies are selected on both ampicillin (Amp) and kanamycin (Kan) plates. The design is such that kanamycin selection should allow us to measure recombination, whereas ampicillin selection should allow us to measure end-joining.

Preparation of DNA for Transfection into Mammalian Cells—M5neoS and M3neoAP are methylated in vitro with

1 The abbreviations used are: bp, base pairs; Amp, ampicillin; Kan, kanamycin.

2 P. Rouet, F. Smih, and M. Jasin, unpublished results.
the cytosine methylase, M. SssI. Although of bacterial origin, the SssI methylase mimics the mammalian methylcytosine transferase, methylating all cytosine residues within the dinucleotide CpG (22). Methylation is monitored by resistance of plasmid DNA to digestion by HpaII, which has a CCGG recognition sequence (Fig. 3). Methylated substrates are not cleaved by HpaII, whereas unmethylated substrates are cleaved to smaller fragments. Methylation appears to be highly efficient by this criteria.

Prior to transfection of DNA into mammalian cells, we monitored the transformation efficiency of the methylated plasmid DNAs in E. coli. DNA was electroporated into E. coli strain DH12S, which is defective in the restriction of methylcytosine containing DNA. Transformation of the parental neo plasmid resulted in almost 2 × 10^9 colonies on either Amp or Kan plates, for an overall transformation efficiency of approximately 4 × 10^9 colonies/μg (Table I). The efficiency was the same whether or not the DNA was methylated.

Transformation of M5neo/S, either methylated or unmethylated, resulted in an approximately 500-fold reduction in the number of Amp^R transformants. This is a result of powerful exonuclease activities present in bacterial cells, which degrades incoming linear DNA. The resulting transformants are likely due to a low level of repair of the broken DNA molecules by the bacterial cells. No Kan^R colonies are obtained with M5neo/S, since the neo gene contains a 3' truncation.

The other recombination substrate, M3neo/AP, transforms bacteria to Amp^R to an even lower level than M5neo/S, possibly due to the close proximity of the AatI site to the start of the amp gene. Thus, M3neo/AP plasmids which have recircularized may have defective amp genes. Alternatively, differences in the overhangs produced by restriction digestion may contribute to the lower transformation efficiency of M3neo/AP. As with M5neo/S, M3neo/AP does not give rise to Kan^R colonies due to truncation of the neo gene.

M5neo/S and M3neo/AP were also mixed prior to transformation into bacteria. Although Amp^R colonies were obtained at approximately the same level as that seen for M5neo/S alone, no Kan^R colonies were obtained, indicating that recombination between the two substrates is very inefficient during bacterial transformation. These results indicate that the bacterial transformation assay will be highly sensitive for the detection of recombination products and that cytosine methylation will not affect the outcome of the assay in bacteria.

Transfection of Methylated DNA into COS1 Cells—COS1 cells were transfected via electroporation with the methylated and unmethylated recombination substrates M5neo/S and M3neo/AP and with the positive control neo. After an incubation of 4 h, extrachromosomal DNA was recovered from the cells and subjected to Southern analysis. This allowed us to monitor the recovery of DNA and the retention of methylation. A similar amount of DNA was recovered whether or not the DNA was methylated.

To determine if CmepG, DNA methylated with methylase SssI, AP, AatI/PstI; S, SphI; H, HindIII.

**TABLE I**

| DNA                  | CmepG | Amp^R | Kan^R |
|----------------------|-------|-------|-------|
| Mneo                 | +     | 1.8 × 10^6| 1.8 × 10^6|
| M5neo/S              | +     | 1.7 × 10^6| 1.6 × 10^6|
| M5neo/S + M3neo/AP   | −     | 3.3 × 10^3| 0     |
| M3neo/S              | +     | 3.2 × 10^3| 0     |
| M5neo/S              | −     | 3.1 × 10^3| 0     |
| M3neo/S              | +     | 3.2 × 10^3| 0     |
| M3neo/AP             | −     | 20    | 0     |
| M3neo/AP             | +     | 8     | 0     |
transformation of the plasmids (Table I). For M5neo it is approximately 14-fold higher, whereas for M3neo it is more than 7000-fold higher. These results indicate that the COS1 cells are able to rejoin the broken DNA ends more efficiently than the bacterial cells and that this end-joining is unaffected by CpG methylation of the plasmid DNA.

The DNA that was transfected into COS1 cells was first cleaved by the restriction endonucleases and then methylated. Since some of the CpG dinucleotides are located within or adjacent to the restriction sites, it is possible that these sites may remain unmethylated. To rule this out, we also performed experiments in which the methylation was performed prior to the restriction digestion. Nearly identical results were obtained in this experiment to those shown in Table II (data not shown), proving that the methylation at the ends does not reduce end-joining efficiencies.

As mentioned above, the difference between the M5neo/S and M3neo/AP plasmids in end-joining efficiencies in bacteria is likely due to the close proximity of the AatII site to the ampicillin gene in M3neo. Alternatively, the particular restriction enzyme cleavage for the two plasmids may play a role. However, following COS1 cell transfection there is little difference between the two plasmids in the generation of AmpR colonies. These results indicate that the joining of ends differs mechanistically between mammalian cells and E. coli.

Individual recombination and end-joining products were analyzed by preparing plasmid DNA from bacterial colonies and subjecting them to restriction analysis. For the recombination products, plasmids were prepared from 30 KanR colonies derived from COS1 cotransfection of M5neo/S and M3neo/AP for both the methylated and unmethylated samples (Fig. 5). Similar results were obtained. Most plasmids were identical to the positive Mneo control (R), indicating that recombination had occurred within both the neo gene and plasmid backbone sequences. A small number of plasmids (RE), 1 or 2 out of 30, had undergone recombination only within the neo gene. DNA end-joining had occurred to join the molecules within the plasmid backbone.

To examine end-joining products, plasmids were prepared from 10 KanR/AmpR colonies from the cotransfection. One class of end-joining products (SE) had precisely rejoined the cohesive SphI ends of M5neo/S. This class consisted of three plasmids derived from the unmethylated sample and one plasmid from the methylated sample. The more numerous class of end-joining products (SE2) was derived from DNA end-joining of the AatII/PstI plasmid backbone fragment of M3neo/AP. This class consisted of seven plasmids from the unmethylated sample and nine plasmids from the methylated sample. Based on restriction analysis, most of the plasmids in this class contained only a limited modification of the AatII/PstI ends. AatII has a 3’ ACGT overhang and PstI has a 3’ TGCA overhang. The rejoin-
ing of these two heterologous ends is not unexpected, given the promiscuity of end-joining in mammalian cells (2).

Direct Examination of Recombination and End-joining Products—Bacterial transformation is sensitive yet indirect for assaying recombination and end-joining processes in mammalian cells. To directly monitor recombination and end-joining in COS1 cells, recovered plasmid DNA was analyzed by Southern blot analysis. A variety of products are expected in addition to those already observed by plasmid recovery (Fig. 6A). Recombination between M5neo/S and M3neo/AP can occur within either the neo gene homology (RE) or the plasmid backbone sequences (ER) or both (R). Similarly, end-joining can occur at the ends of the neo gene homology (ER), the plasmid backbone (RE), or both (EE). In addition, intramolecular end-joining products are also possible. The two M3neo/AP fragments can recircularize to form 3E1 and 3E2, although the latter is not detected since it does not hybridize to the neo probe. M5neo/S can recircularize to form 5E. (Although not illustrated, similar junctions can also be formed in these three cases by intramolecular end-joining of two identical fragments.) One other intramolecular junction is shown. This is formed by end-joining the two neo fragments from M5neoS and M3neo/AP in opposite orientations (E).

For Southern analysis, DNA is cleaved with HindIII and BamHI (Fig. 1) and probed with a neo gene fragment (Fig. 6B). No recombination or end-joining products are detected without prior transfection of DNA into mammalian cells (Fig. 6B, “C” lanes). However, a variety of products are detected upon transfection of either the methylated or unmethylated DNAs into COS1 cells (Fig. 6B, “T” lanes). Cotransfection of M5neoS and M3neo/AP results in formation of both recombination and end-joining products. Recombination occurred within the neo gene (R and RE products) for about 5–10% of the input substrates and is similar in both the methylated and unmethylated samples. The Southern assay gives an apparently higher level of recombination than does the bacterial transformation assay. This difference is due to the nature of the two assays. Southern analysis directly examines the product of recombination within the neo gene. Bacterial transformation to kanamycin resistance requires a second event (recombination or end-joining) within the plasmid sequences, in addition to recombination within the neo gene, so as to generate a closed circular product. This requirement for a second event lowers the measured level of recombination in the bacterial transformation assay.

As with the recombination product, end-joining products are also detected at similar levels in both the methylated and unmethylated samples. The intramolecular products (ER/EE and E) are only detected upon cotransfection of M5neoS and M3neo/AP, whereas the intramolecular product 3E1 is detected in both the cotransfection and the transfection of M3neo/AP alone. The intramolecular product derived from M5neoS is not separated from the input linearized M5neoS in the HindIII/BamHI digest shown in Fig. 6B. However, this product is detected using other restriction digests (data not shown).

**DISCUSSION**

We demonstrate that mammalian cytosine methylation has little or no effect on extrachromosomal recombination in tissue culture cells. End-joining processes also are unaffected by CpG methylation. These results were obtained using a highly sensitive bacterial transformation assay and were confirmed by...
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Recombination in these substrates likely occurs via the single strand annealing pathway (4, 5). This pathway has the requirement that both recombination substrates be cut at or near the regions of homology (26). The DNA ends may then provide an entry site for an exonuclease such that single strands are exposed for annealing. Mechanistically, our results suggest that CpG methylation has little or no effect on the exonuclease and strand annealing activities. A second pathway for homologous recombination, double strand break repair (27) has also been shown to be operational in mammalian cells (28–30). In this pathway, a DNA substrate containing a double strand break is repaired from an unbroken homologous DNA template after strand invasion, conserving both partners of the recombination event. We cannot rule out that CpG methylation may have an effect on this pathway. Experiments that we have performed in which only one of the recombination substrates is broken and the other is introduced as a supercoiled plasmid have shown no difference between methylated and unmethylated substrates.3 However, considering the nonconservative nature of most recombination events between transfected DNAs (31), these results are inconclusive regarding the double strand break repair pathway.

Previous work has shown that CpG methylation and the accessibility of loci to site-specific recombinases are interrelated. A transgene locus that can undergo V(DJ) recombination has been identified that is refractory to recombination when methylated yet accessible for recombination when unmethylated (32). CpG methylated minichromosomes are also inaccessible for V(DJ) recombination (33). Since the inhibition of V(DJ) recombination is apparent only after replication of the minichromosomes, the interpretation of these results is that CpG methylation does not directly affect recombination, but rather results in an altered chromatin structure upon replication which inhibits V(DJ) recombination (33). In addition, demethylation of endogenous T cell receptor and immunoglobulin gene loci has been shown to occur upon activation of lymphocytes which leads to T cell receptor recombination and Ig class switching, another site-specific recombination event (34). Thus, although we find that methylation has no effect on the homologous recombination of extrachromosomal DNA, methylation may yet to be found to exert effects on chromosomal DNA by altering the accessibility of DNA to recombination machinery.

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