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Inheritance of DNA methylation in microinjected eggs of *Xenopus laevis*

(DNA replication/*Hpa* II methylase/hemimethylated DNA)

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ABSTRACT **Plasmid DNA methylated at *Hpa* II sites was injected into eggs of *Xenopus laevis*. The methylated DNA replicates extrachromosomally, and the methylated state of the *Hpa* II sites is inherited through replication. Unmethylated sites remain unmethylated in progeny molecules. To test whether replication is necessary for new methylation to occur, DNA methylated on one strand only was injected and unreplicated DNA was selected for analysis. Methylation was copied onto a previously unmethylated strand in the absence of replication but less efficiently than in its presence. These experiments show that the individual methylated site contains enough information for inheritance of the methylated state and that inheritance of methylation does not require integration of the DNA into the chromosome.**

In higher eukaryotes the only known modification of DNA is methylation of cytosine, predominantly in the sequence 5′-CpG (for review, see refs. 1 and 2). This provides a signal in the DNA that can be varied in different cell types, without altering the base sequences. In principle, such a modification could be used to control functions such as replication or transcription of DNA.

Evidence is accumulating that, in eukaryotic DNA, genes being transcribed are undermethylated, although undermethylation has not yet been shown to cause transcription (1, 2). During development, groups of cells become committed to following certain developmental pathways, and one level at which a developmental program may be inherited after cell division may be by a mechanism such as inheritance of a state of gene methylation. It is known that, in vertebrates, sperm DNA, and the DNA of early embryos, is highly methylated (1). One possible mechanism for differentiation is that a selective loss of methyl groups from particular genes may contribute to determining a developmental pathway. The selective nature of such a mechanism might reside in selective demethylation with passive maintenance of other methylated sites. When a symmetrically methylated sequence $\begin{matrix}{}^{m}\text{CpG}\\ \text{Gp}^{m}\text{C}\end{matrix}$ is replicated, the parental strand will retain the methyl group and this may be copied onto the daughter strand so that $\begin{matrix}{}^{m}\text{CpG}\\ \text{GpC}\end{matrix}$ becomes $\begin{matrix}{}^{m}\text{CpG}\\ \text{Gp}^{m}\text{C}\end{matrix}$ and the methylation state is maintained.

By examining the methylation pattern in *Xenopus* ribosomal genes, Bird (3) has shown that a methylated site is always methylated on both strands, which suggests that methylation is inherited after replication. Pollack *et al.* (4) and Wigler *et al.* (5) have tested whether methylation states are inherited by such a mechanism by introducing methylated DNA into cultured cells and determining the methylation pattern many cell generations later, after it has been integrated into the chromosome. These studies showed that a pattern of methylation can be passively maintained over many cell generations, although in many cases methylation can be lost.

I have studied the inheritance of methylation over a limited number of replications by introducing methylated DNA molecules into a cell in which they replicate extrachromosomally. Bacterial plasmid DNAs were methylated *in vitro* with the prokaryotic enzyme *Hpa* II methylase, which methylates a subset of CpG sequences, those of the form C-C-G-G. The DNA was microinjected into embryonic cells, the eggs of *Xenopus laevis*, which replicate injected DNA (6). By using injected plasmid DNA, I addressed the following questions: (*i*) Does methylation have a role in controlling DNA replication? (*ii*) Does unmethylated DNA become methylated? (*iii*) Is the pattern of methylation inherited? Two further points related to the inheritance of methylation were also tested: (*i*) Is methylation inherited solely on those sequences that were methylated in the injected DNA or do nearby CpG sequences also become methylated? (*ii*) Is DNA replication necessary for new methylation to occur? To study the necessity for DNA replication in the methylation process, DNA methylated in only one strand (hemimethylated DNA) was injected to test whether the unmethylated strand assumes the methylation pattern of the complementary strand. Finally, it is known that sperm DNA is highly methylated, but the methylation state of maternal DNA is difficult to establish. After activating the egg, maternal DNA was labeled by replication in the presence of labeled precursors and its methylation state was determined in the absence of sperm DNA.

MATERIALS AND METHODS

Plasmid pAT153 DNA (a derivative of pBR322) (7) was prepared by the procedure of Birnboim and Doly (8) scaled up to liter quantities of culture. The DNA was purified on a CsCl/ethidium bromide gradient (9). Double-stranded (replicative form) mCet1S DNA, which contains a nematode tRNA gene (10), was a gift of C. Dingwall. Radioactively labeled single-stranded DNA was prepared by growing JM101 cells infected with the M13 phage mCet1S in medium containing $^{32}P_i$ and purifying the phage DNA (10).

Methylation. *Haemophilus parainfluenzae* cells were a gift of M. Carmen (Microbiological Research Establishment, Porton Down, England). *Hpa* II methylase was prepared by the method of Mann and Smith (11). Methylation reactions were carried out in 250 μl of 4 μM *S*-adenosylmethionine (SAM)/50 mM Tris·HCl, pH 7.5/20 mM 2-mercaptoethanol/10 mM EDTA containing 50 μg of DNA and 100 units of enzyme. After 1 hr at 37°C, more SAM and enzyme were added and the reaction was continued for a further hour. The sample was de-

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proteinized by extraction with phenol/chloroform (1:1), phenol was removed by four extractions with ether, and the DNA was concentrated by precipitation with ethanol. An aliquot was exhaustively digested with *Hpa* II at this stage and, if any digestion was detectable, the methylation was repeated.

Construction of Hemimethylated DNA. Supercoiled methylated mCet1S DNA was linearized so that it could be denatured. Ten micrograms of methylated DNA was digested with 100 units of *Bam*HI (New England BioLabs) in 20 μl of 50 mM NaCl/10 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$ and then precipitated with ethanol. Five micrograms of linear methylated DNA was mixed with 7.5 μg of circular single-stranded DNA labeled with ^{32}P *in vivo* to 10^6 cpm/μg in 200 μl of 10 mM Pipes, pH 6.4/5 mM EDTA. The DNA was denatured by boiling for 2 min. The solution was adjusted to 0.3 M NaCl and renatured for 1 hr at 65°C. The DNA was then subjected to electrophoresis on a 1% low-gelling-temperature agarose gel, and the band corresponding to nicked circular DNA was located by ethidium bromide staining and excised. Of the various products of hybridization, only hemimethylated DNA has the nicked circular form, since it arises from circular ^{32}P-labeled virion DNA and linear methylated DNA. DNA was extracted from the gel by melting at 65°C followed by two extractions with phenol at room temperature, concentration by three extractions with butan-2-ol and four extractions with ether, and precipitation with ethanol. The pellet was dissolved in 100 μl of 0.3 M NaCl/10 mM Tris·HCl, pH 7.9/1 mM EDTA and chromatographed on a 1-ml column of LKB Ultrogel AcA 34 in the same buffer. The DNA was precipitated with ethanol and taken up in 50 mM NaCl/10 mM Tris·HCl, pH 7.9/1 mM EDTA for injection. DNA produced in this way was biologically active in injection experiments; it was not toxic to eggs or oocytes, and it was fully active in transcription of the tRNA gene [as assayed by Cortese *et al.* (10)].

Microinjection. Eggs were injected as described by Gurdon (12) except that the first irradiation was omitted. Oocytes were injected as described by Gurdon (13). DNA was extracted and subjected to electrophoresis on agarose gels or centrifuged on CsCl gradients (6). Acid soluble-radioactivity was removed from the samples prior to centrifugation by chromatography on LKB Ultrogel AcA 34.

Restriction Enzyme Digestion. DNA extracted from eggs was purified for digestion by one of three methods: centrifugation on CsCl gradients followed by dialysis and precipitation, chromatography on LKB Ultrogel AcA 34 as described above, or electrophoresis in low-gelling temperature agarose gels as described above. Carrier tRNA was added to ensure efficient precipitation steps. DNA was digested for 2 hr at 37°C by *Hpa* II or *Msp* I (New England BioLabs) according to the manufacturer's instructions in a 50-μl mixture containing 10 units of enzyme and 1 μg of unlabeled DNA, which served as an internal control for complete digestion. DNA was concentrated by precipitation with ethanol prior to electrophoresis on a 1.4% agarose gel.

Analysis of Hemimethylated DNA After Injection. To assay the methylation state of the ^{32}P-labeled strand alone, this strand must be separated from the methylated unlabeled strand by denaturation and hybridized to unmethylated DNA before assaying with *Hpa* II. DNA extracted from eggs was mixed with a 1,000-fold excess of unmethylated mCet1S DNA (2.5 μg). Prior to denaturation, the closed circular DNA was linearized with *Eco*RI [made by the method of Rubin and Modrich (14)] so that the strands would separate and not snap back together. The linear DNA was ethanol precipitated, dissolved in 200 μl

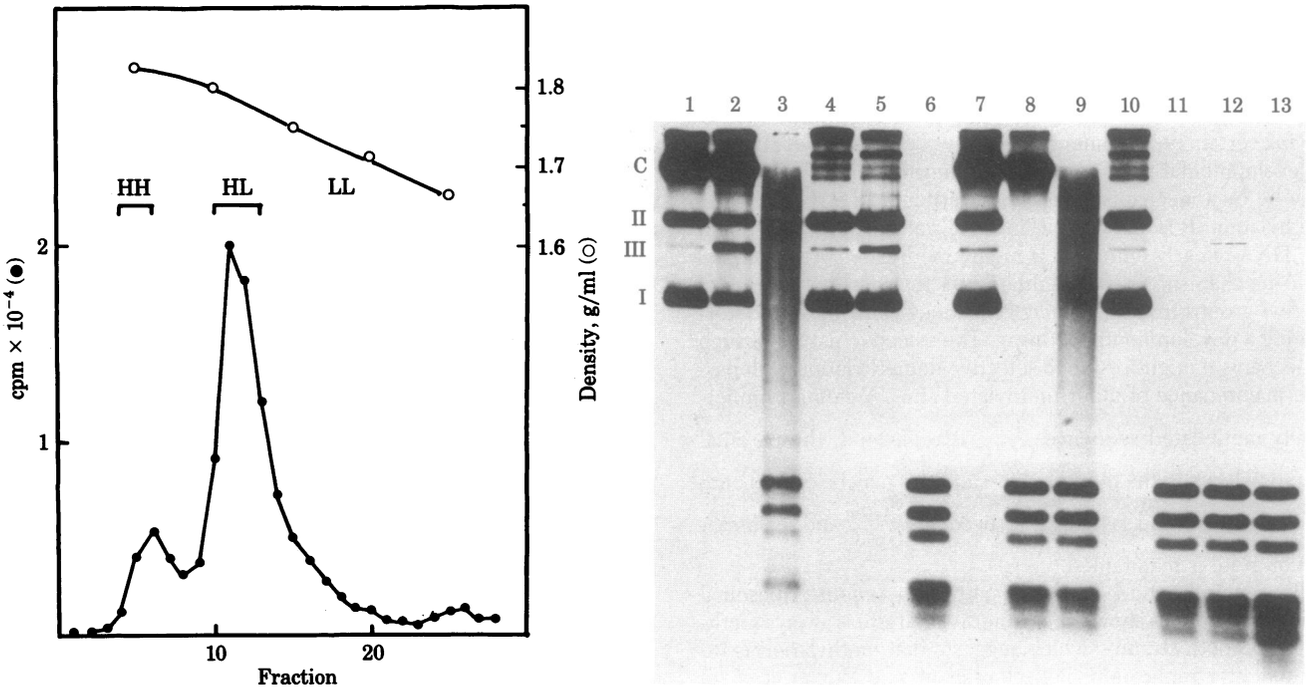

FIG. 1. (*A*) Fractionation of replicated DNA on a CsCl equilibrium gradient. Plasmid pAT153 methylated at *Hpa* II sites was injected at a concentration of 50 μg/ml with deoxybromouridine triphosphate (7.5 mM) and [α-^{32}P]dATP (4 μCi/ml; 1 Ci = 3.7 × 10^{10} becquerels). DNA extracted from 40 eggs was fractionated and assayed by Cerenkov radiation. Heavy·heavy (HH) and heavy·light (HL) fractions were pooled for analysis by restriction enzymes. (*B*) Analysis of replicated DNA by restriction enzyme digestion. HH (lanes 1–3 and 7–9) and HL (lanes 4–6 and 10–12) DNAs were isolated from eggs injected with methylated (lanes 1–6) and unmethylated (lanes 7–12) DNA and subjected to electrophoresis on a 1.2% agarose gel either undigested (lanes 1, 4, 7 and 10) or after digestion with *Hpa* II (lanes 2, 5, 8, and 11) or *Msp* I (lanes 3, 6, 9, and 12). The band marked "C" in the HH fractions arises from the egg chromosomes and is discussed below. Plasmid DNAs: I, supercoils; II, nicked circles; III, linear molecules. The complete *Hpa* II digest of pAT153 is shown in lane 13.

of 10 mM Pipes, pH 6.4/10 mM EDTA, denatured by boiling, and renatured as described above. Aliquots taken immediately after boiling and after renaturation were analyzed on agarose gels to ensure that each step was complete. The renatured DNA was then analyzed by digestion with *Hpa* II or *Msp* I as described above.

RESULTS AND DISCUSSION

Methylated DNA Is Replicated. The replication of methylated plasmid DNA was compared with that of unmethylated DNA in a density-labeling experiment. The DNA was injected into eggs with a radioactive precursor and a density label. Prokaryotic DNA, which is not methylated at CpG sequences, has previously been shown to replicate after injection by this assay (6). The result of an experiment using pAT153 DNA methylated at C-C-G-G sequences by *Hpa* II methylase is shown in Fig. 1*A*. The appearance of discrete peaks of radioactivity at the densities of hybrid heavy·light molecules and heavy·heavy molecules shows that the DNA has replicated (6). Unmethylated DNA that was injected into eggs under similar conditions was replicated to a similar extent (not shown). These experiments therefore suggest that methylation of this kind of sequence does not prevent the DNA from initiating DNA replication.

The Methylation Pattern of Injected DNA Is Inherited. Inheritance of DNA methylation in the egg can be studied by injecting methylated DNA and assaying the molecules produced by replication for the presence of methyl groups. Plasmid DNA was methylated *in vitro* with *Hpa* II methylase. Methylation on one or both strands protects the sequence from digestion with *Hpa* II. Therefore, if *Hpa* II fails to cut a site, the site is assumed to be methylated. To show that the site is still present and that the DNA is accessible to restriction enzymes, digestion is carried out with *Msp* I in parallel. *Msp* I cuts DNA at the same sequence (C-C-G-G) as *Hpa* II but is not sensitive to methylation of the internal cytosine.

The result of an experiment in which methylated or unmethylated DNA was injected into eggs with radioactive and dense precursors is shown in Fig. 1*B*. Radioactive DNA was isolated from density gradients (see Fig. 1*A*), and the heavy·light and heavy·heavy fractions were subjected to electrophoresis either undigested or after digestion with *Hpa* II or *Msp* I.

If unmethylated DNA is injected into eggs, it remains unmethylated. Analysis of such DNA shows that heavy·heavy pAT153 DNA is completely digested to low molecular weight fragments by *Hpa* II (Fig. 1*B*, lane 7). The radioactivity in the HH fraction that is undigested by *Hpa* II and migrates at the position of chromosomal DNA (indicated by "C" in Fig. 1*B*) and the smear in the *Msp* lane of unmethylated HH DNA results from replication of maternal DNA and will be discussed below.

Analysis after injection of methylated pAT153 DNA shows that the heavy·light material is not cut by *Hpa* II (Fig. 1*B*, lane 5) and the radioactivity comigrates with uncut material (lane 4). This is expected if the parental (light) strand retains its methylation pattern and does not test whether the pattern is inherited on the new (heavy) strand. With heavy·heavy material, however, in which both strands are new, the question of whether methylation is inherited can be addressed directly. It can be seen from Fig. 1*B* that the methylated DNA gives rise to methylated progeny; *Hpa* II fails to cut heavy·heavy DNA to low molecular weight fragments (lane 2). Therefore, methylation at the *Hpa* II site is inherited after replication in the egg. The controls with *Msp* I show that the sites are still present in the molecule, and *Msp* I cleaves both heavy·heavy and heavy·light DNA into small pieces (lanes 3 and 6). However, the methylation inheritance is not complete. Long exposure of the gel whose autoradiograph is shown in Fig. 1*B*, lane 2, reveals a ladder of bands produced by partial digestion of some of the replicated material (not shown). There are no digestion products that appear preferentially and, therefore, it is likely that the failure to methylate is not selective at any particular site. One possible trivial explanation for failure to methylate would be that the starting DNA was not methylated on both

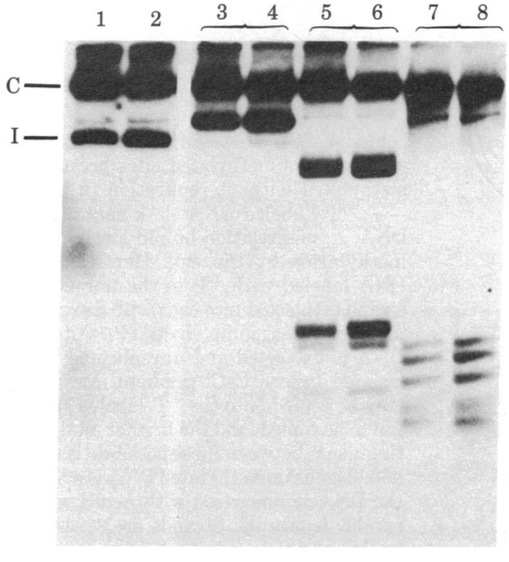

FIG. 2. DNA does not become methylated at new sites. Heavy·heavy DNA isolated from eggs injected with unmethylated (lanes 2, 4, 6, and 8) or methylated (lanes 1, 3, 5, and 7) pAT153 DNA was digested with enzymes [*Ava* I (lanes 3 and 4), *Hae* II (lanes 5 and 6), and *Hha* I (lanes 7 and 8)] sensitive to CpG methylation. Lanes 1 and 2: undigested DNA. I, supercoiled DNA; C, chromosomal DNA. *Ava* I cleaves the plasmid once, leading to a linear molecule (form III) that migrates more slowly than supercoiled DNA.

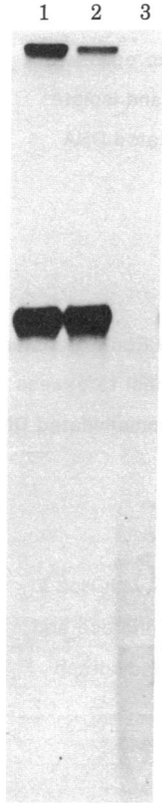

FIG. 3. Methylation of maternal chromosomes. Eggs irradiated at the vegetal pole were injected with [α-^{32}P]dATP (5 mCi/ml). DNA from five eggs was loaded on a 0.6% agarose gel either undigested (lane 1) or after digestion with *Hpa* II (lane 2) or *Msp* I (lane 3). Maternal chromosomal DNA is too large to be resolved by the gel and it moves on electrophoresis as a single band at the exclusion limit of the gel.

strands at every site; however, this possibility is ruled out by the demonstration that sites methylated on one strand only are not present at levels necessary to account for the results. To test for such half-methylated sites, the methylated DNA was denatured and hybridized to ^{32}P-labeled unmethylated DNA and this was digested with *Hpa* II so that any unmethylated sites on starting DNA would give rise to radioactive partial digestion products. Even in grossly overexposed autoradiographs, there was no evidence for such unmethylated sites.

Another possible explanation for incomplete methylation is that the egg is being injected with excessive amounts of DNA that, when replicated, saturate the mechanism used to methylated DNA. This is difficult to exclude; however, one argument against the possibility is that the amount of DNA replicated is closely similar to the amount of chromosomal DNA normally made in fertilized eggs in the same time.

The efficiency of inheritance of methylation can be calculated from the proportion of molecules linearized by *Hpa* II (estimated by microdensitometry of the autoradiograph) and the number, 22, of *Hpa* II sites in pAT153. The efficiency is >99% per replication. Although this does not lead to a large loss of methylation over the two cycles of replication being studied here, clearly over many generations methylation might gradually be lost from the DNA. This phenomenon has been observed by Pollack *et al.* (4) and Wigler *et al.* (5) in cultured cells also. Several explanations are possible. One is that the efficiency of inheritance of methylation depends on chromosomal location. Alternatively the efficiency of inheritance may depend on a cooperative effect of methyl groups on the parental strand. In these experiments, only the *Hpa* II sites (≈1/16 of the available CpG sites) in the DNA are methylated.

Hpa II sites are the only sites at which methylations occur on injected DNA that has undergone replication. The results of an experiment in which heavy·heavy DNA from eggs injected with DNA methylated at *Hpa* II sites or with unmethylated DNA was cut with *Ava* I, *Hae* II, and *Hha* I, which are sensitive to CpG methylation (15), are shown in Fig. 2. DNA produced by replication of either methylated or unmethylated injected DNA was cut to the same extent; demonstrating that these sites remain unmethylated. Methylated sites are therefore inherited accurately, and one mechanism whereby methylation could be inherited is ruled out: i.e., that the methylase recognizes new DNA opposite a methylated strand and moves along it, methylating all possible CpG sites.

These results show that a pattern of methylation imposed *in vitro* on prokaryotic DNA by a prokaryotic enzyme can be inherited when the circular DNA replicates in the egg. These results, like those of Pollack *et al.* (4) and Wigler *et al.* (5) suggest that an important unit of inheritance of methylated sites is the individual methylated site.

Methylation of Maternal DNA. Although it is known that sperm DNA is highly methylated, it is difficult to obtain enough

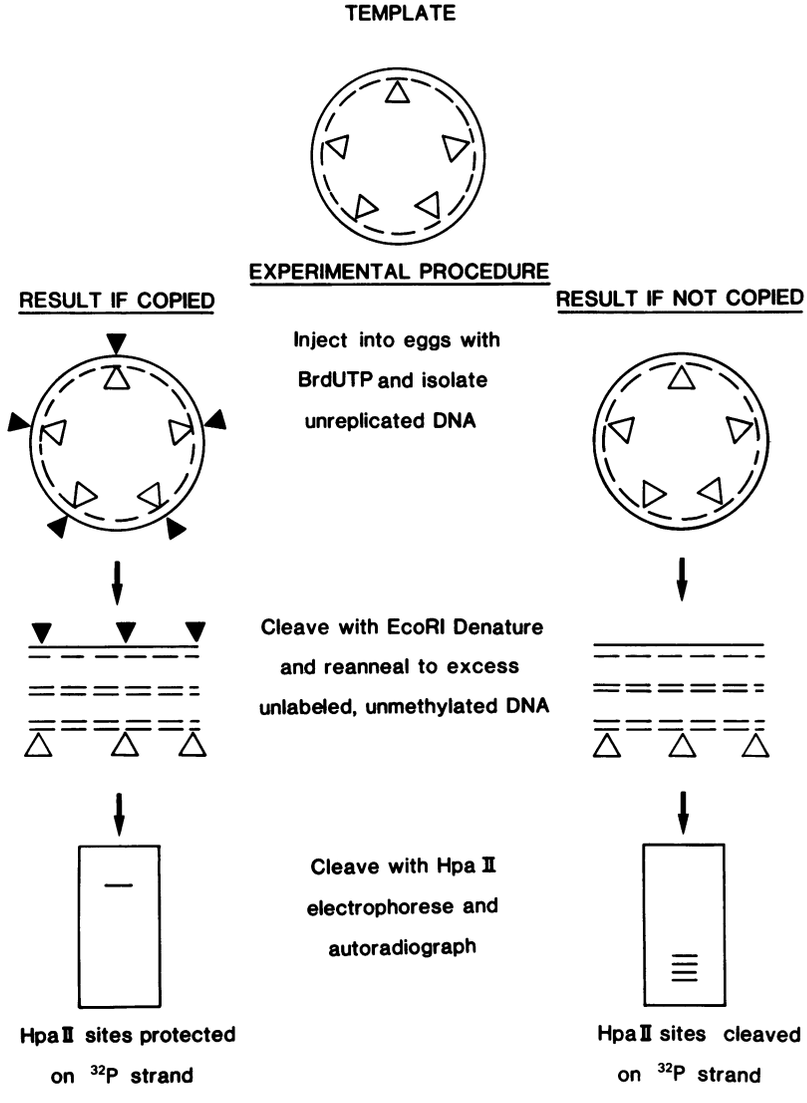

FIG. 4. Schematic of experiment to find whether hemimethylated sites become fully methylated without replication in the egg. ——, ^{32}P-Labeled DNA; ----, nonradioactive DNA; △, methylation on old strand; ▲, new methylation by the egg. Hemimethylated DNA labeled with ^{32}P in the unmethylated strand is injected into eggs with deoxybromouridine triphosphate (BrdUTP). After 5 hr, DNA is extracted and unreplicated DNA is isolated from a CsCl gradient. Methylation may be copied onto the ^{32}P-labeled strand or fail to be copied, and the further analysis distinguishes between these possibilities. Excess homologous unmethylated DNA is added, and the DNA is linearized with *Eco*RI so that it can be denatured. Strands are separated by boiling and then rehybridized so that the radioactive DNA is now hybridized to unmethylated DNA. The DNA is digested with *Hpa* II. This will now fail to cleave *Hpa* II sites in the radioactive DNA that become methylated, whereas DNA that did not become newly methylated will now be digested to small fragments. The distribution of radioactivity after gel electrophoresis therefore distinguishes between new methylation or failure to methylate.

DNA from oocytes or eggs to assess the state of methylation of maternal DNA. However, when radioactive label is injected into unfertilized eggs, the maternal chromosomes replicate and become labeled (16). When replication of injected DNA is being studied, chromosomal replication is reduced to background levels by irradiation of the egg chromosomes at the animal pole by UV light (6). However, if the unfertilized eggs are irradiated at the vegetal pole, the label incorporated into maternal chromosomal DNA can be detected at a stage at which it is difficult to obtain enough DNA to visualize by other means. Analysis of this DNA by *Hpa* II and *Msp* I cleavage shows that the maternal chromosomes are highly methylated during early cleavage and, because methylation is inherited, it is likely that the chromosomes of the unfertilized egg are also methylated (Fig. 3).

Does Copying of Methylation Require DNA Replication? Since DNA methylation is a postreplicative event, the substrate for methylation in the cell is a hemimethylated molecule: i.e., one that is methylated in only one strand. The question of whether a methylation pattern can be copied only immediately after replication or at other times in the cell cycle can be addressed by the injection experiment shown schematically in Fig. 4. A hemimethylated DNA molecule was constructed from methylated double-stranded mCet1S DNA and unmethylated ^{32}P-labeled single-stranded mCet1S DNA. In the hemimethylated molecules, the unmethylated strand was ^{32}P labeled so that any new methylation on this strand in the egg could be detected as described below. The DNA was injected into unfertilized eggs with deoxybromouridine triphosphate and, after 5 hr incubation, DNA was extracted and fractionated by centrifugation to equilibrium on a CsCl gradient to isolate light·light DNA for further analysis. The hemimethylated DNA starts as form II (nicked circles; Fig. 5, lane 1) and is not susceptible to cleavage by *Hpa* II (lane 3), though it can be cleaved to small fragments by *Msp* I (lane 4). After injection, the DNA is ligated and assembled into chromatin so that, after extraction and purification of unreplicated DNA, some of the radioactivity migrates in the position of form I DNA (supercoils; lane 5). This material is still resistant to *Hpa* II but can be cleaved to completion by *Msp* I (lanes 6 and 7). Therefore, the DNA has not been demethylated. However, because the nonradioactive parental strand is still methylated, this analysis does not show whether the previously unmethylated ^{32}P-labeled strand has become methylated. To assay for new methylation, the methylated nonradioactive DNA strand is replaced by hybridization with excess unmethylated nonradioactive DNA as shown in Fig. 4. The susceptibility of the radioactively labeled DNA to *Hpa* II now reflects its state of methylation. The radioactive DNA hybridized to unmethylated DNA migrates as form III (linear molecules; Fig. 5, lane 8). This DNA is susceptible to *Msp* I (lane 10) as expected. However, when digested with *Hpa* II, it proves to be partially resistant to the enzyme (lane 9). This is not due to incomplete activity of the enzyme, since unlabeled DNA added to the reaction mixture and visualized by ethidium bromide fluorescence after gel electrophoresis was digested to completion (not shown). The conclusion therefore is that some but not all of the methylated sites in hemimethylated DNA are copied onto the unmethylated strand in the absence of replication.

These results suggest that there are two levels of methylation activity or possibly two separate methylation activities. Highly efficient methylation occurs only after replication. Perhaps this activity is closely associated with the complex that replicates DNA. A lower level of methylase activity can operate at other times. In neither case, however, was a methylation pattern copied fully onto a new strand of DNA, as discussed earlier, and, as suggested by experiments using transfer of DNA onto cultured cells, additional factors are probably operating to maintain a completely methylated state in DNA. Nevertheless, it is striking that methylation at *Hpa* II sites in the plasmid is a sufficient signal for inheritance of such methylated sites at high frequency, even when the DNA replicates extrachromosomally.

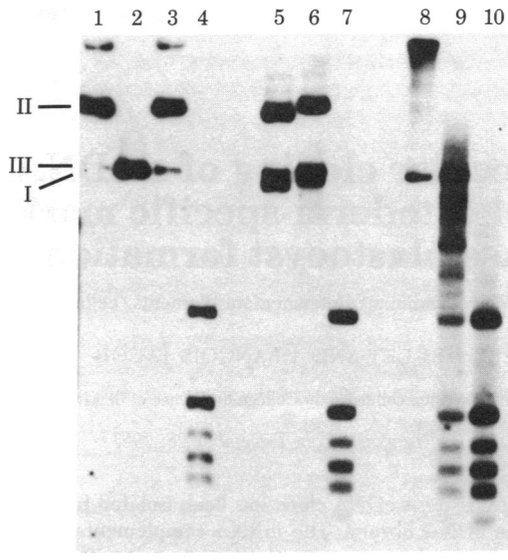

FIG. 5. Hemimethylated DNA injected into eggs becomes partially methylated on the previously unmethylated strand. Lanes: 1, undigested purified circular hemimethylated DNA (^{32}P-labeled single-stranded M13 DNA hybridized to unlabeled methylated DNA); 2, the same DNA was digested with *Eco*RI, which cuts the DNA once; 3, lane 1 DNA digested with *Hpa* II; 4, lane 1 DNA digested with *Msp* I; 5, undigested DNA extracted from eggs injected with hemimethylated DNA; 6, lane 5 DNA digested with *Hpa* II; 7, lane 5 DNA digested with *Msp* I; 8, undigested DNA extracted from eggs injected with hemimethylated DNA was cut with *Eco*RI and the ^{32}P-labeled strand was hybridized to unmethylated DNA; 9, lane 8 DNA digested with *Hpa* II; 10, lane 8 DNA digested with *Msp* I.

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