Fitting Methylation into Development

Molecular biologists now think that methylation is a secondary mechanism that may help establish DNA structures to turn genes on and off

Walter Gilbert of Harvard University walked into a recent NIH meeting* on DNA methylation as a skeptic, but an open-minded one. He came, he says, not because he is a "methylologist" but because he was curious. For years now, molecular biologists have been hoping to show that cells use the simple procedure of moving methyl groups around on DNA to turn genes on and off during development. The question is, Is methylation really that fundamental a mechanism or does it have a less central role?

The answer is that methylation no longer looks like the solution to the mystery of development. This is not to say that it is irrelevant to development but rather that it almost certainly is not a primary mechanism. On the other hand, secondary mechanisms can certainly be important. Says Arthur Riggs of Beckman Research Institute of the City of Hope, "the definitive experiment [showing methylation is crucial for development] has already been done." So the story of methylation continues and as its true function unfolds, researchers are being led closer and closer to an understanding of what actually goes on in development and how it occurs.

Methylation of DNA is part of the ancient history of molecular biology. It was first noticed in 1948 by R.D. Hotchkiss, soon after it was discovered that DNA is the genetic material. In methylation, a certain number of cytosines, one of the DNA bases, is enzymatically converted to the chemically distinct 5-methylcytosine and this 5-methylcytosine acts just like a fifth DNA base. (When they were determining the structure of DNA, James Watson and Francis Crick chose to ignore this fifth base, although they knew of its existence.) The fact that 5-methylcytosine is enzymatically created and thereafter acts like a fifth base is what makes methylation so alluring a mechanism to explain differentiation.

But the differentiation connection was slow in coming. For more than 25 years after Hotchkiss' discovery of methylation, most molecular biologists either disregarded the phenomenon or focused instead on the methylation of RNA. Then in 1975 both Riggs and, independently, Robin Holliday of the National Institute for Medical Research in London, proposed a critical hypothesis: methylation is heritable, passed on from generation to generation as cells divide. It was known that methylation always occurs at sequences where cytosines are followed by guanine, denoted CpG. That means that opposing DNA strands are symmetrically methylated. The cytosine of the newly synthesized DNA strand is methylated as soon as the sequence is made.

The implication, says Riggs, is that methylation "acts like a secondary genetic code." Once a CpG sequence is methylated, it stays that way. And if a CpG pattern is not methylated, it will remain that way. In fact, adds Aharon Razin of the Hebrew University in Jerusalem, cells have been followed for hundreds of generations and their methylation patterns remain stable. It seemed possible that cells use methylation to turn genes off permanently during development.

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About 90 percent of the DNA in a mammalian cell is permanently turned off during development and the particular gene sequences that are inactivated vary from cell to cell. However that is accomplished, it must be heritable. When a cell that is committed to be a muscle cell divides, for example, its progeny must also be committed to be muscle cells and so the same DNA sequences must be turned off in them. This is the attraction of methylation. It offers a way for cells to turn off genes and keep them turned off from cell generation to generation.

As soon as they recognized methylation's potential, molecular biologists began looking for correlations between methylation patterns and gene activity. It got to a point, says Razin, at which "everyone who had a [cloned] gene was looking at methylation patterns." And patterns did emerge. In roughly one-third of the 30 or so genes examined so far, the genes are active only if they are undermethylated. Other genes, the so-called housekeeping genes which are turned on all the time in all cells, lack methyl groups at their initiation sequences, the 5' ends, which is also in keeping with the hypothesis that methylation of genes turns them off and keeps them turned off.

But, says Riggs, "God was unkind." In about 20 percent of the genes examined, there seems to be no good correlation between methylation and gene activity. Worse still, lower vertebrates in general have far less methylation than mammals—20 percent of the lower vertebrate DNA is methylated as compared to 80 percent of mammalian DNA—and the fruit fly *Drosophila* has essentially no methylated DNA at all. Yet, says Gilbert, "lower vertebrates have perfectly nice life cycles. They have blood, they have muscles, and their muscles work. The cynical view is that there is no strong connection [between methylation and gene activity]."

Still, Riggs remarks, perhaps it is asking for too much to propose that methylation is the primary means of turning genes off during differentiation. "I think it is a mistake to try and answer how important methylation is. We know it is significant and that it is a system used by mammals. Mammals control genes over eight orders of magnitude—there is 10^8 more growth hormone message in the rat pituitary than in the liver. The *lac* repressor [which controls a bacterial gene] controls over only three orders of magnitude. Mammals have 10^5 more capacity. So I think it is probable—it almost has to be—that mammals have multiple mechanisms to control genes. One of them is methylation."

Moreover, Riggs continues, there is strong evidence that manipulating methylation can turn mammalian genes on or off. The evidence that so impresses him involves X chromosome inactivation. Female mammals have two X chromosomes, but only one is active. Early in

*"The Chemistry, Biochemistry, and Biology of DNA Methylation," held 17 to 19 April at the National Institutes of Health.

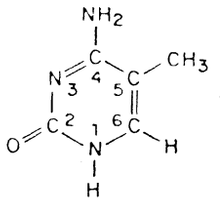
embryo development, at about the time the embryo implants in the uterine wall, one entire X chromosome turns off. Moreover, genes from this inactive X chromosome do not function well in DNA-mediated gene transfer. The genes from the active X chromosome work 100 times better. "This tells you that the DNA is changed," Riggs says.

A few years ago, Peter Jones of the University of Southern California and Lawrence Shapiro and T. Mohandes of the University of California at Los Angeles found that the drug 5-azacytidine can reactivate genes of inactive X chromosomes. This drug, Jones had previously shown, is a potent inhibitor of methylation. "People had been trying to do this [reactivate X chromosomes] for 20 years," Riggs points out. Then Shapiro and his colleagues showed that genes from reactivated X chromosomes function in gene transfer. The evidence from X chromosome activation that methylation plays a role in turning genes on and off is, Riggs remarks, "about as close to definitive as you can get in biology."

And there are other striking experiments as well. Razin and his associate Lea Reshef, for example, gave 5-azacytidine to rat embryos and looked at an enzyme, PEPCK, that normally becomes active only at birth. They had already established that the PEPCK gene sequentially becomes demethylated as fetal rats mature and that this demethylation seems to be what allows the gene to be turned on at birth. When the rat embryos were treated with the drug, their PEPCK genes were demethylated and the enzyme was turned on and stayed on.

Not everyone, however, is convinced by experiments using 5-azacytidine. Although it indisputably prevents methylation, no one has proved that it has no other effects. Gilbert, for one, describes experiments with this drug as "a way of hitting a cell with a hammer and hoping that something pops out."

But the evidence does not end with 5-azacytidine. Researchers have recently done careful experiments with promotors, which precede genes and allow them to be turned on, showing that when a promotor is methylated it does not function. Walter Doerfler of the University of Cologne, for example, reports that methylation of viral promotors determines whether the promotors function in gene transfer experiments. He took adenovirus promotors and hooked them to a test gene, chloramphenicol acyltransferase. Then he showed that if the promotor is methylated, the promotor-gene sequence does not function in gene transfer, whereas if it is not methylated the sequence does function. "Methylation of the promotor determines gene activity," he concludes.



Structure of 5-methylcytosine

But if methylation plays a role in determining gene activity, what sort of role could that be? In answering that question, researchers are hampered by a fundamental missing link. They have not yet found the enzyme that originally adds methyl groups to DNA when genes are permanently turned off during development. What they have found instead is enzymes that keep methyl groups on when cells divide—"maintenance enzymes," as they are called. The lack of the initial methylating enzyme, says Gilbert, "leaves us with a feeling that there is a whole missing side to the story of methylation."

Yet, notes Razin, it is not too surprising that the initial methylating enzyme has not been found. "The enzymes we have were isolated from somatic tissues, where they are supposed to have only maintenance activity. What is missing is what happens when the methylation patterns are changing. That occurs in a very short time during embryogenesis and you have to know when to look. We didn't look in the right place. We looked under the light."

Razin presented at the meeting a description of experiments designed to look closely at just what does happen when methylation patterns change. The idea is to get a little farther from the light and see what happens when cells start to differentiate. Razin and his colleagues began with leukemia cells, which are undifferentiated blood cells, and added any of a number of chemicals that cause these cells to differentiate within 5 days. What they saw was "genome-wide hypomethylation." The cells lost methyl groups transiently and quickly. It indicates, says Razin, "that hypomethylation is active, not passive." And just as rapidly, the cells established a new methylation pattern.

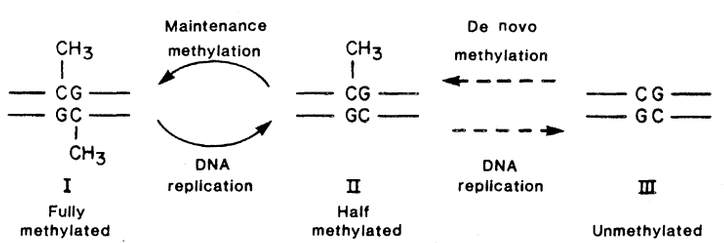
Razin proposes that what methylation does is lock nucleosomes into position on the DNA. It is believed that DNA is wound around protein balls like beads on a string and that the control regions of active genes are those not wound up in these nucleosomes. The DNA in nucleosomes has four times more methyl groups than DNA in regions between them. Razin's proposal is that methyl groups stop nucleosomes from sliding and that when methyl groups are removed, the cell can go from one state of differentiation to another.

When a new state of differentiation is established, Razin further suggests, the nucleosomes are fixed in position by what he calls "determinator proteins." Then the DNA is methylated to lock this pattern in, whereupon the determinator proteins are no longer required.

Not only does this model explain why methylation might be a secondary rather than primary controller of gene expression, but it also explains the problem of *Drosophila*, according to Razin. If an organism does not have very many cell cycles, it can lock its nucelosomes into position by making determinator proteins every cycle. *Drosophila* has few cell cycles and so it may not need the refined and efficient methylation system used by higher organisms.

So the picture that is now emerging is that methylation may be at the bottom of a hierarchy of controls that determine which genes are turned off and how and that its role may be to lock the final stage into place. As Gilbert remarked at the close of the meeting, "I would view the methylation controls as probably the very bottom level, the last level of control that is used to shut genes off. Some of that level is now much clearer from discussions at this meeting."

—GINA KOLATA



How methylation occurs

It is believed that DNA is originally methylated early in development and then, each time the DNA replicates, the methylation pattern is carried along. After methylated DNA replicates, enzymes add methyl groups to the newly synthesized DNA strand.