Articles

The Inheritance of Epigenetic Defects

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Evidence from many sources shows that the control of gene expression in higher organisms is related to the methylation of cytosine in DNA, and that the pattern of methylation is inherited. Loss of methylation, which can result from DNA damage, will lead to heritable abnormalities in gene expression, and these may be important in oncogenesis and aging. Transformed permanent lines often lose gene activity through de novo methylation. It is proposed that epigenetic defects in germline cells due to loss of methylation can be repaired by recombination at meiosis but that some are transmitted to offspring.

THE PROPERTIES OF GENES IN HIGHER ORGANISMS CAN BE studied on two levels: first, the mechanism of their transmission from generation to generation, which is the central component of genetics and is well understood, and second, their mode of action during the development of the organism from the fertilized egg to adult, which is very poorly understood. The changes in gene activity during development are generally referred to as epigenetic, a term first introduced by Waddington (*1*). Thus, epigenetic switches turn particular genes on or off during the developmental process, producing either transient changes in gene activity or a permanent pattern of activities.

The classical studies on nuclear transplantation in *Xenopus* by Gurdon and his collaborators (*2*) made it unlikely that differentiation involved irreversible genetic changes, such as mutation or chromosome rearrangements. The differentiation of cells that synthesize immunoglobulin provides at least one exception to this, since in such cells DNA sequences are rearranged prior to the formation of the structural gene (*3*). This situation is unusual, since the rearrangement is one part of the mechanism that creates antibody diversity, and it is more generally believed that highly specific protein-DNA interactions are responsible for epigenetic changes in gene activity. One essential feature of these interactions may depend on the postsynthetic chemical modification of bases in DNA. It was first pointed out by Scarano (*4*) that the significance of DNA modification mechanisms is that they provide a molecular basis for the inheritance of a particular pattern of gene activities. Several types of modification are possible, but attention recently has been focused on the importance of 5-methylcytosine (5mC) in the control of gene activity. It has been proposed (*5*) that the particular pattern of unmodified or modified cytosines could be inherited, if there were a maintenance methylase that recognized the hemimethylated DNA formed after replication and that methylated the nascent strands; such an enzyme would not act on sequences that contained nonmethylated cytosine.

The general hypothesis for the control of gene expression by methylation depends on the specificity of DNA-protein interactions. It is supposed that there are specific protein transcription factors that recognize methylated or nonmethylated sequences and interact with RNA polymerases. Cytosine methylation occurs in CpG doublets in the DNA of higher organisms. Since this does not by itself provide specificity, it is proposed that surrounding sequences are essential for protein recognition. This implies that most 5mC residues have nothing to do with gene expression and have some other function. Clusters of CpGs are not usually methylated in HTF islands adjacent to structural genes, and there are grounds for believing that these may have a regulatory role (*6*).

The proposal for epigenetic controls based on DNA methylation (*5*) attempted to explain two major features of the developmental program: (i) the segregation of gene activities and (ii) the stability of cells that are finally differentiated or, in the case of cell determination, that are destined to differentiate at some later stage of development. Stable differentiated cells may have ceased division, as have neurons or muscle cells, or be capable of prolonged growth, as seen, for example, in the serial subculture of fibroblasts, lymphocytes, or glial cells. Examples of the inheritance of determined cell states include many types of stem cells and the imaginal disk tissue of *Drosophila*. It has been suggested that some specific DNA-protein interactions are themselves heritable (*7*), but in this review the discussion is confined to mechanisms based on DNA modification.

Several lines of evidence have strongly implicated 5mC in the control of gene expression in higher organisms [for reviews, see (*8*)]. There are now many examples where gene transcription is correlated with the absence of methylation at one or more sites in the promoter or sometimes other regions of the gene, and in cells not expressing the gene these sites were found to be methylated. However, in most of these studies it is not clear whether methylation is a cause or an effect of gene inactivation. Evidence that gene inactivation is a result of methylation comes from experiments in which the γ-globin gene with a methylated promoter is cotransfected with a selectable thymidine kinase gene. It can be shown in individual clones that the globin gene is present but not expressed, whereas the same gene with a nonmethylated promoter is expressed (*9*). There is little information about methylation switch mechanisms that bring about different cell types, although it has been shown that muscle cells can specifically remove methyl groups from transfected actin genes and thereby activate them (*10*). Also, genomic sequencing has shown that vitellogenin synthesis induced by estradiol is specifically associated with the removal of methyl groups at the estradiol-receptor binding site at the 5′ end of the vitellogenin gene (*11*). Some of the strongest evidence that methylation is important for the epigenetic control of gene transcription comes from experiments that show that the pattern of methylation is inherited through DNA synthesis and cell division.

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Heritability of the Pattern of DNA Methylation

Evidence that the distribution of 5mC in DNA is heritable comes from direct and indirect experiments. In the globin transfection experiments just mentioned, the DNA remains in a methylated or nonmethylated state during the growth of individual clones (*9*). However, in these and other transfection experiments the fidelity of maintenance of methylation is not very high, since 1 to 5% of methyl groups are lost per cell division (*12*). In other experiments, methylated DNA was injected into *Xenopus* eggs and allowed to replicate (*13*). In this case the fidelity of maintenance was greater, since less than 1% of 5mC was lost per replication.

When retrovirus DNA is injected into preimplantation mouse embryos, it can be integrated in the chromosome and is de novo methylated and inactivated (*14*). This inactivation persists through development into the adult, and it can be shown that the methylation of this DNA has been faithfully maintained. The examination in female mammalian cells of X-linked genes, such as those coding for hypoxanthine-guanine phosphoribosyltransferase (HGPRT), phosphoglycerate kinase, and glucose-6-phosphate dehydrogenase, has shown that the inactive state of the X chromosome is correlated with methylation in HTF islands that are associated with these genes, whereas these islands are not methylated on the active X chromosome (*15*). Since the active and inactive states are stably inherited, the pattern of DNA methylation in these regions of DNA must also be very stably maintained. It has been shown that in transfection experiments the wild-type $HGPRT^+$ gene on an inactive X chromosome will not produce $HGPRT^+$ colonies in an $HGPRT^-$ recipient, whereas the same DNA from cells with $HGPRT^+$ on the active X chromosome will produce such colonies (*16*). These experiments also indicate that the presence or absence of DNA methylation is maintained in the recipient cells.

Gene Reactivation by 5-Azacytidine

The cytosine analog 5-azacytidine (5-aza-CR) has a nitrogen atom substituted for a carbon atom at the site of methylation of cytosine. It is also a potent inhibitor of DNA transmethylase, which is known to bind the analog, possibly by a covalent linkage (*17*). In many studies it has been shown that cells treated with 5-aza-CR or 5-aza-deoxycytidine (5-aza-CdR) have a significantly reduced level of 5mC in their DNA. Genes that are not transcribed can very often be reactivated by a single exposure to 5-aza-CR or 5-aza-CdR. This is true for latent retroviruses, and in several instances it has been shown that the activation of the virus by 5-aza-CR is associated with changes in DNA methylation (*18*). The very low spontaneous frequency of activation of genes on an inactive X chromosome can be increased at least a thousandfold after 5-aza-CR treatment (*19*).

Many rodent cell lines that lack particular enzymes or proteins are very stable and appear at first sight to have classical mutations in structural genes. However, 5-aza-CR treatments can induce massive reactivation of such genes, with as many as 10 to 30% of the survivors recovering enzyme activity, which represents about a millionfold increase over the spontaneous reversion rate. Mutagens or agents that damage chromosomes do not have these effects, and 5-aza-CR is itself only a weak mutagen in mammalian cells (*20*). Silent genes that have been reactivated by 5-aza-CR are listed in Table 1 (*21–38*). These results provide strong evidence that genes are often inactivated in permanent cell lines by methylation, that the methylation is very stably inherited, and that 5-aza-CR results in demethylation of sites important for the control of gene activity. In several cases this has been confirmed by examination of the gene in question by means of methylation-sensitive restriction enzymes (Table 1, *21–38*).

Heritable Changes in DNA Methylation

The results summarized in Table 1 do not explain the origin of the enzyme-deficient strains. In many rodent cell lines derived from primary diploid cells, recessive mutations can be obtained at surprisingly high frequency, which led Siminovitch (*39*) to propose that they are "functionally hemizygous." Elimination of chromosomes or parts of chromosomes by rearrangement could lead to haploidy of a considerable proportion of the genome and facilitate the isolation of mutants. An alternative possibility is that these lines have a de novo methylase activity that will inactivate one of the two gene copies, if such inactivation does not have a selective disadvantage. Normal mutations can then be introduced into the active gene, and the 5-aza-CR will reactivate the silent gene (Fig. 1). In some cases, such as

Table 1. Cell lines that lack specific enzymes or proteins and that often regain a normal phenotype after growth in 5-azacytidine.

Cell line and organism	Altered gene	Phenotype	Enzyme or protein reactivated	Reference
W7 Mouse	MT^-	Cadmium sensitive	Metallothionein	(*21*)*
CHO Hamster	MT^-	Cadmium sensitive	Metallothionein	(*22*)* (*23*)*
V79 Hamster	tk^-	Bromodeoxyuridine resistant	Thymidine kinase	(*24*)
CHO Hamster	tk^-	Bromodeoxyuridine resistant	Thymidine kinase	(*22*)*
L5178Y Mouse	tk^-	Bromodeoxyuridine resistant	Thymidine kinase	(*25*)
L-61-M Mouse	tk^-	Bromodeoxyuridine resistant	Thymidine kinase	(*26*)
FEL Mouse	tk^-	Bromodeoxyuridine resistant	Thymidine kinase	(*27*)
L tk^- Mouse	HSV†	Bromodeoxyuridine resistant	Thymidine kinase	(*28*)* (*29*)
CHO Hamster	ODC^-	Requires putrescine	Ornithine decarboxylase	(*30*)
FA32,Fu5 Rat	OCT^-	Requires arginine	Ornithine carbomyltransferase	(*31*)
IR45 Rat	asp^-	Requires asparagine	Asparagine synthetase	(*32*)
CHO Hamster	asp^-	Requires asparagine	Asparagine synthetase	(*33*)
CHO Hamster	pro^-	Requires proline	Pyrroline-5-carboxylate synthase and ornithine aminotransferase	(*34*)
V79 Hamster	glu^-	Requires glutamine	Glutamine synthetase	(*35*)
GH_3 Rat	$rPRL^-$	Prolactin deficient	Prolactin	(*36*)
HeLa H23 Human	$hgprt^-$	6-Thioguanine resistant	Hypoxanthine-guanine phosphoribosyltransferase	(*37*)
CHO Hamster	*xrs*	Radiation sensitive	Unknown: deficient in DNA double-strand break repair	(*38*)

*Reactivation shown to be associated with loss of 5-methylcytosine. †Herpes simplex virus.

auxotrophy or cadmium sensitivity (metallothionein-deficient), the phenotype arose spontaneously during cell culture, which may be the result of de novo methylation of both gene copies without selective disadvantage. There is also evidence that genes that have been activated in permanent cell lines can again be inactivated during growth, presumably by de novo methylation (*27, 28, 40*). When the total level of 5mC in C3H 10T½ cells was progressively reduced by sequential treatments with 5-aza-CdR, the methylation level was slowly restored after cessation of these treatments (*41*).

It has been proposed that heritable changes in gene activity due to DNA modification should be referred to as epimutations to distinguish them from classical gene mutations, which are due to changes in DNA sequence (*38*). Thus, de novo methylation and 5-aza-CR introduce forward and reverse epimutations in mammalian cell lines.

If DNA methylation is essential for the normal controls of gene activity during development, it follows that defects in methylation could have severe phenotypic consequences in diploid somatic cells.

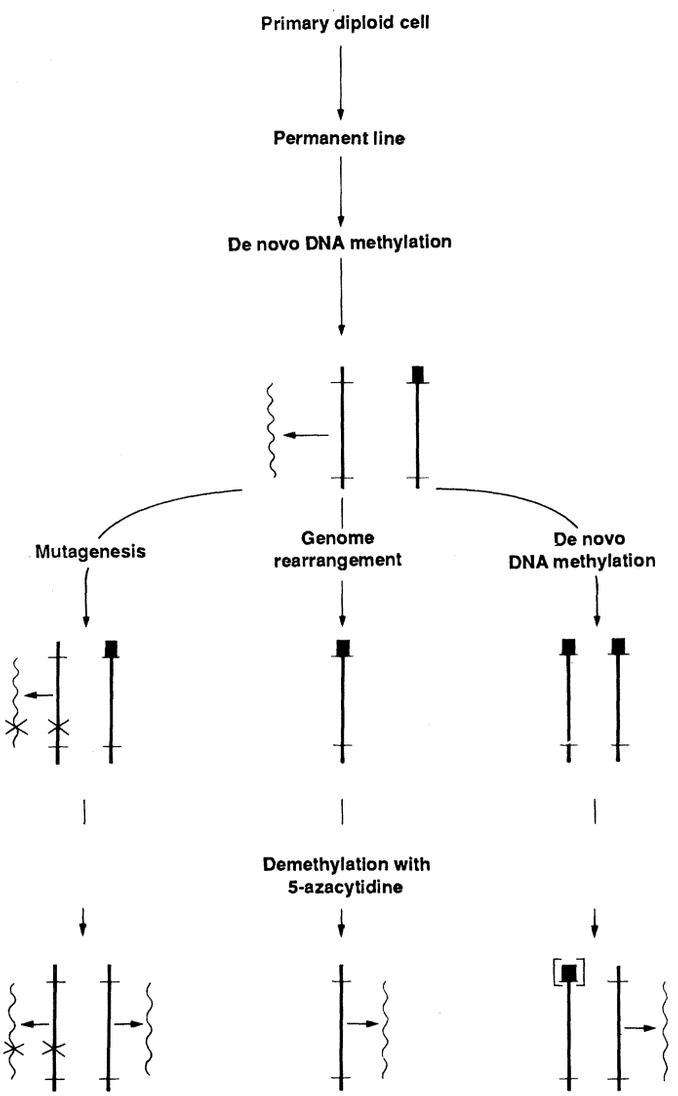

Fig. 1. The origin of inactive genes in permanent cell lines. Functional hemizygosity (*39*) can be due to the inactivation of one copy of an autosomal gene by de novo DNA methylation [(*38*) and see text]. Subsequently mutagenesis inactivates the second copy and 5-aza-CR reactivates the silent copy (left). Other possibilities include loss of an active gene after chromosome rearrangement or nondisjunction, followed by de novo methylation of the remaining copy (center), or de novo methylation of both gene copies, if this confers no selective disadvantage (right).

So far, there is no evidence for the shutting off of genes by de novo methylation in these cells, apart from X-chromosome inactivation. However, it is possible that DNA damage could bring about such changes (*36, 37*). In contrast, there is evidence for the continual loss of DNA methylation during the growth of diploid cells (*42*). Loss of methyl groups could occur in several ways, including the following:

1) The maintenance methylase may be less than 100% efficient, so that there is a small constant probability that hemimethylated DNA will persist from one S phase to the next and subsequently give rise to nonmethylated DNA, which is no longer a substrate for the enzyme. [However, it is also likely that not all methylation sites are maintained with equal fidelity (*43*). Critical sites may be more stable, possibly through the activity of a more efficient sequence-specific maintenance enzyme.]

2) Abnormal bases in DNA produced spontaneously or by external agents may inhibit the action of the maintenance methylase and lead to the formation of nonmethylated sequences. It has been shown that damage to hemimethylated DNA, especially alkali-labile lesions and single-strand breaks, strongly inhibits the activity of mouse spleen methyltransferase (*44*). Although ultraviolet (UV) light was not active in these experiments, a subsequent study demonstrated that cells with a silent metallothionein gene produced a high frequency of cadmium-resistant revertants after UV treatment and that 30 to 40% of these synthesize metallothionein message and had become demethylated in the region spanning the gene (*45*).

3) Damage in DNA will often be repaired by the filling of excision tracts, and these will initially be hemimethylated. If such tracts are formed immediately in front of a replication fork or in the template strand of newly synthesized DNA, then nonmethylated sequences will be produced (*46*). It is also possible that postreplication recombination repair of gaps opposite a lesion will produce hybrid DNA regions that are not methylated.

4) Methylation is known to occur fairly soon after nascent strains of DNA are synthesized, and it is possible that free ends are important for normal methylase activity in vivo. If this is so, then agents that introduce single-strand gaps or breaks in DNA may inhibit normal methylation. The maintenance methylase might bind to these free ends, and the efficiency of normal methylation at replication forks would be correspondingly decreased. In other words, the enzyme would be "titrated out" by the presence of single-strand breaks. The special properties of x-rays in inducing epigenetic defects will be described below.

5) The methylation of DNA depends on the presence of adequate quantities of 5-adenosyl methionine. Thus, any treatment that inhibits its formation, such as ethionine, is liable to induce the loss of methyl groups. Ethionine can reactivate a silent gene (*24*).

Whereas cultured diploid cells progressively lose methylation, permanent lines retain a constant level. In such lines it may well be that loss is balanced by de novo methylation. The general pathways for changes in methylation are outlined in Fig. 2, but it must be emphasized that almost nothing is known about the enzymic mechanism for de novo methylation in vivo, and whether or not it is in any way sequence specific.

The Frequency of Cellular Transformation in Rodent and Human Cells

The frequency of neoplastic transformation of rodent and human cells is vastly different. It is well known that primary cultures of mouse or rat fibroblasts will consistently give rise to permanent lines, which can subsequently acquire all the usual properties of fully transformed cells (*47*). The spontaneous frequency of immortaliza-

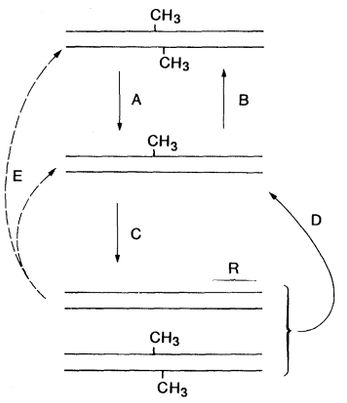

Fig. 2. Pathways of demethylation and methylation of cytosine in DNA. Pathway A: DNA replication. Pathway B: Maintenance transmethylase, which is not sequence specific. Pathway C: Failure of maintenance or excision repair of DNA damage means the replication fork can lead to loss of methylation [(*46*) and see text]. Pathway D: Nonmethylated promoter-recombinator sequences (R) may initiate recombination and the formation of heteroduplex DNA (*79, 84*), which is an essential intermediate in crossing-over and gene conversion (*81, 83*). Hemimethylated hybrid DNA is a substrate for maintenance methylase. Pathway E: De novo DNA methylase acts on nonmethylated DNA. It is not known whether or not it is sequence specific.

tion of hamster cells has been estimated to be 1.9×10^{-6} (*48*). It is also well established that a variety of carcinogens can increase the transformation frequency substantially, by at least 100-fold (*49*). However, primary cultures of human fibroblasts are extremely refractory to transformation. In innumerable life-span experiments in many laboratories, there have been no reports of spontaneously arising permanent transformed lines, even though such lines would be readily selected from senescent populations of diploid cells. If we take all experimentation into account, it can be concluded that the spontaneous frequency of transformation is 10^{-9} or less. Also, treatment of human diploid fibroblasts with carcinogens very rarely yields transformed derivatives capable of prolonged growth (*50*).

These observations are not easily compatible with the mutational origins for transformation, since studies of spontaneous and induced mutation (for example, to 6-thioguanine or ouabain resistance) have not indicated that frequencies are different in rodent and human cells (*39, 51*). Recently a method has been developed for measuring mutation to 6-thioguanine resistance in lymphocytes, and it has been shown that the frequencies in human and mouse are similar (*52*).

It was previously suggested that at least one of the steps in cellular transformation may be due to an epigenetic change in gene activity, such as the loss of DNA methylation induced by DNA damage (*46*), and there is now evidence in support of this viewpont (*53*). The frequency of such changes may well vary greatly between species, which makes biological sense when one takes into account the size and longevity of animals and the probability of neoplastic transformation. Humans have about 3000 times as many cells as mice and there is a 30-fold difference in maximum life span. Thus, on a per cell basis the likelihood of a tumor arising is about 10^5 greater in a mouse than it is in a man, and according to the multistage model for carcinogenesis the difference in frequency of each event could be as high as 10^9 (*54*). It is striking that similar large differences in cellular transformation frequency in mice and humans are seen both in vitro and in vivo.

It is therefore possible that cells from large, long-lived species are effectively buffered against epigenetic changes. One possibility would be an increase in the number of methyl groups involved in the control of gene activity. Suppose, for example, that a single 5mC in a promoter region can prevent transcription, and that it can be lost at a frequency of 10^{-3} per cell division; then the existence of two 5mCs in the same region, either of which can prevent transcription, would ensure that the activation of the gene in question would occur with the frequency of 10^{-6}, and so on. This predicts that promoter regions of silent genes would be more heavily methylated in human cells than in rodent cells. The argument that anomalous epigenetic switches in gene activity may be buffered in human cells is supported by experiments with 5-aza-CR. Mouse cells undergo morphological changes when grown in the presence of the analog, but human fibroblasts do not do so (*55, 56*). Chick fibroblasts are also resistant to morphological changes and, like human cells, are refractory to neoplastic transformation in vitro (*56, 57*).

Experiments by Kennedy *et al.* (*58*) have shown that there are at least two steps in the in vitro transformation cells of the C3H 10T½ mouse line. They cloned individual cells that had survived x-irradiation with 400 or 600 rads and found that transformed cells very commonly appeared in such clones, whereas those from untreated cells did not produce any transformants. This experiment showed that x-ray treatment altered most of the surviving cells in such a way that a second rare event occurred in at least one of their descendants. Whereas the second event could have been a mutation or chromosome rearrangement, the first event induced by x-rays could not have been, because it occurred too frequently. It is possible that the x-ray treatment, which would introduce about 5000 single-strand breaks per genome, would inhibit maintenance methylase activity and thereby lead to the loss of 5mC at a significant number of sites. This may have no initial phenotypic effect, but would predispose the cells to alter their phenotype by a subsequent rare event, which could be mutation or, alternatively, another epigenetic change.

Epigenetic Inheritance Through the Germline

In this section a number of examples of inheritance will be briefly reviewed that are not due to the Mendelian transmission of classical mutations. In some cases an epigenetic basis for this inheritance is clear, whereas in others it is supposition. X-chromosome inactivation in female mammals is clearly an epigenetic phenomenon, and there is strong evidence that DNA methylation is involved (*15, 16*). In marsupial mammals the paternal X is inactivated in all cells in female offspring, and in eutherian mammals the paternal X is preferentially inactivated in the extraembryonic tissues. These observations demonstrate that the X chromosomes inherited from the male and female parents are different, a phenomenon usually referred to as chromosomal imprinting. Imprinting is obviously heritable, but is reversed in the germline, possibly at the time an inactive X is reactivated. Imprinting of autosomal chromosomes has also been demonstrated in mice (*59*) and has been known for a long time in a variety of insects (*60*).

Recently evidence has been obtained for transmission of altered methylation patterns through the germline. The controlling element *Ac* (activator) in maize is capable of transposition, and a derivative that has lost transposase activity has been shown to have methylated cytosine in CCGG, CAG, and CTG sequences. This is inherited through sexual crosses, but can revert to active *Ac*, either in the germline or somatic tissue, with loss of methylation but with no indication of a change in base sequence (*61*). Other examples of sexual transmission of heritable changes in DNA modification in maize are also known (*62*). These results suggest that the well-known phenomena of "presetting" and changes in state of maize-controlling elements studied by McClintock (*63*), and paramutation studied by Brink (*64*), may also be due to changes in DNA methylation.

A problem in mouse genetics is the unexpected variation in homozygous inbred animals. Grüneberg and his associates have fully documented the variability of skeletal structures in inbred lines (*65*). The frequency of variation is far higher than that expected from mutation, and it was suggested that a latent virus might be responsible for this variability. An alternative explanation is that the

morphological variation is due to heritable modification of germline DNA. Experiments with mice have shown that x-ray and carcinogenic treatments can result in tumor formation in subsequent generations (*66*). For example, male or female parents treated with x-rays or urethene produce offspring with a greatly increased level of tumors compared to controls. This predisposition to carcinogenesis was inherited as if it were a dominant mutation with 40% penetrance. However, the frequency of induced mutation would have to be extraordinarily high, at least ten times more frequent than dominant skeletal mutations, which are themselves much more frequent than expected from mutation (*65*). Indeed, it can be calculated that the number of loci responsible for delayed carcinogenesis would have to be as large as 400, if it is assumed there is equal sensitivity to x-ray mutagenesis of any of this large number of "tumor gene" loci and seven specific loci that produce visible recessive mutations (*67*). This explanation lacks plausibility. The results are, in fact, similar to those of Kennedy *et al.* (*58*) and immediately suggest that a heritable defect, other than mutation, is often induced in germ cells by the treatment. This defect would predispose the offspring to produce in somatic tissue a further event, or events, leading to tumor formation, the frequency of which, on a per cell basis, could be quite low.

Epigenetic Defects and Aging

If it is assumed there is a given low probability that a methyl group will be lost at each cell division and that de novo methylation either does not occur, or occurs at a lower rate, then it follows the level of methylation should progressively decline. Previous discussions of error theories of aging have concentrated on protein and DNA defects (*68*), but to these should now be added the possibility of epigenetic errors through the loss of methyl groups (*69*). Wilson and Jones (*42*) measured the level of 5mC, using accurate chromatographic procedures, in cultured diploid human, hamster, and mouse cells. They found that the level declined in all these species but most slowly in human cells, which have the longest life span, and most rapidly in mouse cells, which have the shortest. Hamster cells were intermediate, with regard to both longevity and the rate of loss of methyl groups. Thus, the rate of decline of methylation correlates with in vitro life span, at least for these three species. Presumably, the observed rate of loss was dependent on cell division, rather than on chronological time. The division dependence of the Hayflick limit has never been satisfactorily explained in terms of the protein error theory, since nondividing confluent cells are also turning over proteins. However, the steady loss of methylation would provide a satisfactory explanation for this limit. This possibility has been tested with 5-aza-CR and 5-aza-CdR. A single treatment inhibits growth to some extent, but cells rapidly recover and have a normal phenotype and growth rate. However, they retain a memory of the treatment because their growth potential is substantially reduced by premature senescence (*69, 70*). Multiple treatments have a progressively strong effect in shortening life span. These results suggest that the physiological age of the cells is advanced by the loss of methylation induced by 5-aza-Cr. Permanent lines may escape senescence because they appear to have the ability to restore methyl groups by de novo transmethylase activity (*40, 41*).

In mice it has been demonstrated that autosomal DNA inserted into an inactive X chromosome is often itself inactivated. In Cattanach's insertional translocation (*71*), this inactivation can be demonstrated, for example, with the tyrosinase wild-type allele, since it produces an albino phenotype. Cattanach has made the remarkable observation that as the animals age, the degree of inactivation declines in the translocated segment, and the animals become progressively more pigmented (*71*). Histological procedures have also provided direct evidence for reactivation of a normal inactive X chromosome during the aging of mice (*72*). These results could be explained if DNA methylation declined during aging and reactivated silent genes. In other experiments, the methylation of intracisternal A particle chromosomal DNA in liver has shown a highly significant decline during aging (*73*).

It has long been known that whole-body ionizing radiation leads to a reduction in life span. The evidence that this is due to premature aging is compelling (*74*), and the results have therefore often been cited as support for the somatic mutation theory of aging (*75*). However, there are several reasons for believing that somatic mutation is not a primary cause of aging (*76*), and it is therefore possible that the effect of x-rays is due to the introduction of substantial members of epigenetic defects in somatic cells.

This phenotypic effect of epigenetic changes in somatic cells may differ in at least two important respects from mutation in structural genes. First, the effect would be primarily on the regulation of gene activity, rather than the integrity of coding sequences. Thus, loss of methyl groups may most commonly lead to the expression of genes that are usually silent in any particular type of somatic cell. This may be relatively less harmful than the inactivation of an important gene by mutation. Second, genes that are inactive in somatic cells may often have a cluster of 5mC, rather than one 5mC, in the adjacent promoter region. If this is so, the expression of that gene would depend not on the loss of a single methyl group, but on the sequential loss of several. It would be possible, therefore, to have many silent epigenetic defects that would only produce a deleterious phenotype after a given period of time or a given number of cell divisions. In addition, it is not unlikely that regulatory defects, which on their own are relatively innocuous, may interact with other defects that produce a correspondingly more severe phenotype. The changes associated with aging do not usually occur at a steady rate throughout the life span; instead, they accumulate more and more rapidly as the end of the normal life span is approached. This appears to be true of the whole organism, as well as cultured human diploid cells (*77*). A multiplicity of phenotypic effects can be accounted for by a feedback mechanism, such as the Orgelian error catastrophe (*78*), or any multistep mechanism with more than additive interactions between individual small defects.

Meiosis and the Repair of Epigenetic Defects

One essential role for meiosis may be the reprogramming of gametes prior to the formation of fertilized eggs. According to the DNA methylation theory of development, this reprogramming would depend, at least in part, on specific de novo methylation or demethylation of chromosomal DNA. However, it is a reasonable supposition that transmethylase maintenance activity is also essential in germline cells, including meiocytes, to prevent the transcription of genes with specialized somatic functions. The nonprogrammed loss of methyl groups creates a problem for any de novo methylase: on the one hand the methylase would have to be highly sequence-specific, in which case it would not recognize all possible demethylated sites; or it would have to be nonspecific, in which case it would methylate a large number of sites that must not be methylated in meiosis. The complete information required for reprogramming the pattern of DNA methylation is unlikely to reside in a single cell; it is more likely that it occurs in sequential steps in germline cells, or very early in development.

It has been proposed that the recombination at meiosis may play an essential role in the repair of epigenetic defects due to the loss of methylation (*79*). Several series of observations on crossing-over

and gene conversion in eukaryotes are relevant to this possibility. It was pointed out by Thuriaux (*80*) that the map length of genomes in eukaryotic organisms is quite independent of DNA content. Thus, over a 1000-fold range in DNA, the total frequency of crossing-over per meiosis in 12 species is in the same range. He also examined the published data on the size of genes, as determined by fine structure mapping. In fungi, maize, and *Drosophila*, the frequency of intragenic recombination remains surprisingly constant. These observations lead to the almost inescapable conclusion that most recombination in these organisms is confined to structural genes or their immediate vicinity, because, if this were not so, the amount of recombination in a gene in maize would be almost 1000-fold less than in yeast. It is also worth noting in this connection that recombination does not occur in inactive heterochromatin, for instance, in B and Y chromosomes, which do not contain structural genes. Another series of observations in fungi makes it clear that recombination is not randomly distributed throughout a given gene. Most usually it is polarized, with recombination events such as gene conversion occurring much more commonly at one end of the gene than the other. This is usually interpreted to mean that the formation of hybrid DNA is initiated at particular sites at the end of or to one side of a coding sequence (*81*). Such sites have been termed "recombinators," and, if recombination is largely confined to structural genes, then it is reasonable to equate promoter or operator sequences with recombinators (*82*). An important class of epigenetic defect may be the loss of methylation in such sequences. I suggest that nonmethylated promoters have recombinator activity and specifically initiate recombination (*79*). Formation of hybrid DNA would follow, and this DNA would be hemimethylated, since the homologous chromosome is very unlikely to carry an epigenetic defect at the same site. This maintenance enzyme will now methylate the previously unmethylated strands, thus eliminating the defect (Fig. 2). The nonmethylated recombinator could be a substrate for a nuclease, which initiates recombination either by a single-strand or possibly a double-strand break (*83*). The resemblance of such an activity to a bacterial Type 1 restriction enzyme may not be fortuitous.

On the basis of quite different evidence, Catcheside (*84*) has independently proposed that recombination in *Neurospora* is initiated by an enzyme that produces a double-strand cut in the vicinity of a nonmethylated DNA sequence, and that the same methylated sequence is not a substrate for the enzyme.

The Significance of Outbreeding

The possible efficiency of recombination in removing epigenetic defects is, of course, a matter of speculation, but it is a reasonable supposition that in the absence of specific de novo methylation, some defects will not be repaired at meiosis and could therefore be transmitted to the next generation. Defects that are not repaired at meiosis will have properties formally equivalent to mutation, since they are heritable and can have specific phenotypic effects, but there are very significant differences as well. When a mutation is heterozygous, half the offspring will inherit the mutant allele and the other half the wild type. However, when an epigenetic defect is heterozygous, there will be given probability of its removal, or conversion to wild type, by recombination at meiosis. This may throw light on the significance of outbreeding. Assume epigenetic defects arise in the germline at a constant frequency at any of a large number of sites in the genome. According to the hypothesis, there is a given probability that each will be removed at meiosis, but a proportion are transmitted to the next generation. With outbreeding, there will be the same probability that each defect will be eliminated at the next meiosis. Thus, epigenetic defects will be continually arising, but at any subsequent meiosis each is liable to be eliminated. Leaving aside selective forces, the end result will therefore be a steady-state level of defects. This situation is in sharp contrast to inbreeding. In this case, when an epigenetic defect is transmitted to a fertilized egg, there is a 25% chance it will become homozygous in the next generation. Once a defect is inherited, inbreeding through successive generations will result in its becoming homozygous in 25 to 50% of individuals, depending on the likelihood of its removal in heterozygotes at meiosis.

The evolution and maintenance of sexual reproduction in higher organisms pose considerable problems for evolutionary biologists and population geneticists (*85*). The existence of heritable epigenetic defects, which can be removed at meiosis when heterozygous but not when homozygous, could account for inbreeding depression and the advantages of outbreeding and perhaps could help explain the ubiquity of meiosis and sex.

Conclusions: Mutation and Epimutation

Once it is accepted that epigenetic controls of the level of DNA transcription are heritable in cell lineages, then it follows that heritable defects or abnormalities in such controls are possible. The implications of this are far-reaching. Such epigenetic defects may produce a phenotype formally identical to mutation, but there is suggestive evidence that under some circumstances they may be induced at much higher frequencies. Such defects are likely to be the cause of teratogenic abnormalities and a strong case can be made for their role in neoplastic transformation. It is also possible that they are responsible for the loss of cellular homeostasis associated with aging. Epigenetic defects need not be confined to somatic cells, but could also be transmitted through the germline to subsequent generations. It is suggested that meiosis, recombination, and outbreeding play an important role in preventing the accumulation of defects, many of which may initially be phenotypically silent, predisposing the individuals inheriting them, or their descendants, to phenotypic instability such as developmental abnormalities or the onset of tumors.

Mendel's first law states that two alleles in a heterozygote segregate without influencing each other, so the possibility that epigenetic defects might be removed at meiosis contravenes this law. In this regard, such defects are reminiscent of paramutation, discovered by Brink (*64*) in maize, which he defines as "an interaction between alleles that leads to directed, heritable change at the locus with high frequency, and sometimes invariably within the time span of a generation" (p. 129). A similar non-Mendelian interaction between alleles may occur in the fragile X-chromosome syndrome in humans (*86*). It is proposed that the heritable changes based on DNA modification should be designated *epimutations* to distinguish them from classical mutations, which are changes in DNA sequence (base substitution, insertion, deletion, or rearrangement). Mutations are induced by many DNA damaging agents, usually at low frequency, and they are not repairable once fixed in the genome. Epimutations can be induced by treatments affecting DNA modification, often at very high frequency, and they may be repairable when heterozygous. It is suggested that epimutations may occur more frequently in small, short-lived animals than large, long-lived ones, whereas classical mutations seem to occur at similar frequencies in different mammalian species.

Epigenetics is concerned with the strategy of the genes in unfolding the genetic program for development. This strategy is not understood and the lack of a theoretical framework severely hinders experimental advances. The discovery that heritable modification of

DNA is often related to gene expression opens up new possibilities, some of which are explored here. The discussion certainly raises more questions than it answers. For example, are deviations from Mendelian ratios sometimes associated with alterations in DNA methylation? Are demethylating agents such as 5-aza-CR also recombinogenic? What are the rules governing the stability of gene expression in normal and transformed cells, and can it be established that epigenetic defects in gene expression are more frequent in rodent than human cells? Do they accumulate during aging? These and many other related questions could be answered by the use of experimental techniques now available.

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The Jupiter-Io Connection: An Alfvén Engine in Space

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Much has been learned about the electromagnetic interaction between Jupiter and its satellite Io from in situ observations. Io, in its motion through the Io plasma torus at Jupiter, continuously generates an Alfvén wing that carries two billion kilowatts of power into the jovian ionosphere. Concurrently, Io is acted upon by a J × B force tending to propel it out of the jovian system. The energy source for these processes is the rotation of Jupiter. This unusual planet-satellite coupling serves as an archetype for the interaction of a large moving conductor with a magnetized plasma, a problem of general space and astrophysical interest.

SPACE PLASMA PHYSICS IS THE STUDY OF PLASMAS IN THE heliosphere, ranging from the solar interior to the boundary between the extended solar atmosphere and the interstellar medium (*1*). Solar system plasmas, which can be probed directly, provide unique insights into general problems in plasma physics, especially those involving the dilute plasmas commonly found in astrophysics. Historically, one of the intriguing puzzles in this field has been the nature of the processes responsible for the radio bursts from Jupiter at decameter wavelengths. In this article, I review spacecraft measurements made in 1979 in the vicinity of Io, which demonstrate quantitatively that these decameter bursts are powered by the rotation of Jupiter through the intermediary of its satellite Io. These measurements illustrate the central role of in situ observations in understanding the complex physical processes occurring in the large-scale plasma systems found in nature.

The jovian decameter radio bursts were first discovered in 1954 (*2*). Subsequently, and surprisingly, the decameter bursts were found to be strongly controlled by the innermost Galilean satellite Io (*3*). Io is located 5.9 jovian radii (R_J) from the center of Jupiter (R_J = 71,400 km), has a diameter D of 3672 km, and has no known intrinsic magnetic field. Io was found to influence the decameter bursts through its magnetic "flux tube." The Io flux tube (IFT) is that bundle of jovian magnetic field lines that instantaneously thread Io, as illustrated to scale in Fig. 1. A significant fraction of the decameter bursts are found to come from the region in the jovian ionosphere at the ends of the IFT, over 300,000 km away from Io, with the radiation emitted in a highly anisotropic fashion along the surface of cones (*4, 5*). A number of theoretical models were advanced to explain this puzzling phenomenon (*6–13*).

The Voyager Science Steering Group decided in the early 1970s to target the Voyager 1 spacecraft for the IFT, some 20,500 km south of Io (Fig. 1). Voyager observations near the IFT have provided a quantitative understanding of the energy source for the decameter bursts and have revealed the exotic nature of the plasma environment in which Io resides. A detailed analysis of the magnetic field observations near the IFT has been available for some time (*14*), but data from the plasma measurements there have only recently been fully reduced because of the difficulty of the analysis (*15, 16*). As I discuss here, the complete plasma and magnetic field data sets near the IFT show remarkable agreement with the Alfvén wing theory first advanced by Drell, Foley, and Ruderman in 1965 (*17*). To understand the IFT measurements, however, we must first discuss the bizarre plasma environment near Io, since this environment determines the nature of the Jupiter-Io electromagnetic interaction.

The Io Plasma Torus

Io is one of the major plasma sources in the jovian magnetosphere. For reasons still not completely understood, but perhaps related to its active volcanism, Io is accompanied in its orbit by extended clouds of neutral gasses (sodium, potassium, sulfur, and oxygen) that have escaped from its surface (*18–20*). These neutral clouds move at approximately Io's orbital velocity of 17 km/sec and are limited in spatial extent because of the finite lifetime (a few tens of hours) of the neutrals before they are collisionally ionized by

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