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X inactivation, differentiation, and DNA methylation

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Abstract

A model based on DNA methylation is proposed to explain the initiation and maintenance of mammalian X inactivation and certain aspects of other permanent events in eukaryotic cell differentiation. A key feature of the model is the proposal of sequence-specific DNA methylases that methylate unmethylated sites with great difficulty but easily methylate half-methylated sites. Although such enzymes have not yet been detected in eukaryotes, they are known in bacteria. An argument is presented, based on recent data on DNA-binding proteins, that DNA methylation should affect the binding of regulatory proteins. In support of the model, short reviews are included covering both mammalian X inactivation and bacterial restriction and modification enzymes.

In this paper I attempt to explain mammalian X inactivation on the bases of DNA methylation and the properties of bacterial DNA methylases. Since most readers familiar with bacterial methylases may not be familiar with the X inactivation phenomenon, and vice versa, it was thought necessary to review briefly both areas before presenting new models.

The X inactivation phenomenon

Female mammals have two X chromosomes, whereas males have only one, creating a potential gene-dosage differential. In fact, however, this differential does not exist because a mechanism has evolved to maintain gene dosage effectively constant in both sexes. This mechanism, called X inactiva-

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tion, results in one, and only one, active X chromosome per diploid autosomal set of chromosomes. Any additional X chromosomes are genetically inactive, heteropyknotic, and late replicating. The condensed, inactive X chromosome forms the sex chromatin, or Barr body, usually seen in interphase and prophase and located near the nuclear membrane. The inactivation phenomenon (OHNO et al., 1959; LYON, 1961; BEUTLER et al., 1962) is well documented at the cytological and genetic level and is the subject of several recent reviews (OHNO, 1969, 1974; EICHER, 1970; COOPER, 1971; LYON, 1971, 1972; BROWN and CHANDRA, 1973).

For this paper, I will make the following assumptions about X inactivation in eutherian mammals: (1) *Activation*. The key step is activation of one chromosome. Initially, both chromosomes of the zygote are inactive, but by the time of implantation (50 to 400 cells), one and only one X chromosome has been activated. The condensation and late-replicating pattern of one X chromosome can be considered as a secondary consequence of the lack of activation. (2) *Randomness*. On the average, half of the cells of an individual have the maternal X chromosome active and half have the paternal X chromosome active. (3) *Permanence*. Except in the germ line, the initial differentiation event is irreversible. The inactive X chromosome in a particular cell remains inactive in all progeny cells. Inactivation is maintained throughout all successive cell divisions and concomitant DNA replication events. (4) *Totality*. The entire X chromosome is activated or inactivated as a unit. In mice, there is good evidence for a single primary X inactivation center in the X chromosome (RUSSELL, 1963; RUSSELL and MONTGOMERY, 1965; CATTANACH et al., 1970; DREWS et al., 1974). Translocated X chromosome fragments lacking the inactivation center remain active (CACHEIRO et al., 1973). Thus, inactivation apparently spreads from the inactivation center to cover all (or almost all) genes on the X chromosome. Although there may not be unanimous agreement on all these points, I believe the above statements are in keeping with the current consensus. For a more detailed discussion of these points, the reader is referred to the recent reviews listed above.

X inactivation occurs early in development, when the fate of embryonic cells is being determined, and thus can be considered an event in embryonic differentiation. The phenomenon of X inactivation evolved fairly recently, at about the time of emergence of the common precursor of mammals and marsupials (OHNO, 1969). A mechanism for the inactivation of entire chromosomes has also evolved in some insects, e.g., mealy bugs (BROWN and NUR, 1964; CHANDRA, 1971). OHNO (1969, 1973) argues that since evolution

is a very conservative process, seldom using truly new mechanisms, X inactivation probably results from minor variations of the basic mechanisms of gene regulation and differentiation. Thus, there is reason to think that an explanation of X inactivation may throw some light on the mechanisms of differentiation.

Review of present theories

Several attempts have been made to explain X inactivation at the molecular level. However, rather than attempt a comprehensive review, I will select only two types of models that I think are particularly successful in explaining the basic facts of X inactivation. Then I will try to make some generalizations about all models. Finally, I will point out some weaknesses in these models.

The earliest model proposed, the episomal activator model of GRUMBACH et al. (1963), remains one of the most attractive. As illustrated in fig. 1, they proposed that an episomal factor becomes incorporated at a specific receptor

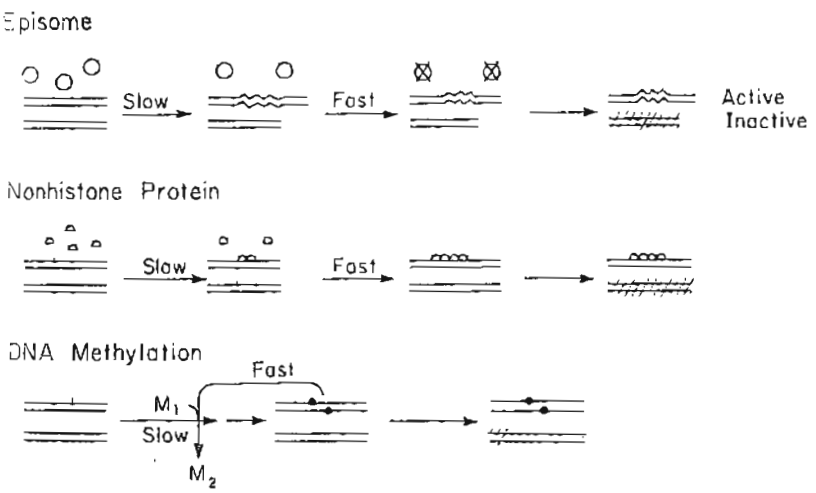

Fig. 1. Molecular models for X inactivation. Model 1 proposes the integration of an episome (circle) into the inactivation center of one X chromosome. The X chromosome with the integrated episome remains active, whereas the other X chromosome becomes inactive. Model 2 proposes that nonhistone proteins keep one X chromosome active. Model 3 proposes that methylation of the inactivation center of one X chromosome causes it to remain active. See text for additional details on all three models.

locus on the X. This leads to both the activation of the X with the integrated episome and also the inactivation of any other unincorporated episomal factors in the cell. It was also necessary to postulate that the episome was removed from the active X during oogenesis or spermatogenesis so that the inactivation would again be random in the next generation.

On the other hand, OHNO (1969, 1973) and LYON (1971, 1972) have proposed models based on sequence-specific DNA-binding proteins (non-histone proteins) that bind at a certain site (the inactivation center), thereby leading to activation of the chromosome. OHNO's model (DREWS et al., 1974; OHNO, 1974) is also illustrated in fig. 1. He proposes that the autosomes produce a limited number of protein molecules designed to bind cooperatively at the inactivation center. The first molecule binds randomly, but because of the high cooperativity, all others bind adjacent to the first molecule on the same chromosome. Later, a generalized X chromosome repressor substance is produced to inactivate any chromosome not having the inactivation center covered by the cooperatively binding activator protein.

What do most of the present models have in common? First, to explain the randomness of inactivation (activation), a single receptor site on the X chromosome is postulated at which some entity binds specifically. Second, most models propose either (a) a *single* entity (protein, episome, or membrane attachment site) or (b) a *slow* initial activation process followed by a fast secondary process. The initial activation step must be slow because if this were not the case, the activation of both X chromosomes often would occur. In the episome models, the slow process is the integration of the first episome; the fast process is the destruction of any extra episomes. In the cooperative-binding protein model, the slow process is the binding of the initial protein molecules; the fast process is the cooperative binding of all other activator proteins adjacent to the first.

What are some of the problems with the present models? The episome activator model of GRUMBACH et al. (1963) can explain most aspects of X inactivation, especially randomness and permanence. It offers no good explanation for the totality of inactivation, but then, none of the present models (including the model to be presented here) offers a convincing explanation of the spread of inactivation to cover the entire X chromosome. Perhaps the major difficulty with episome models is that they have not stimulated biochemical experimentation. These models also have the defect of being rather unappealing as general models for differentation; to have the expression of most genes controlled by the integration of episomes seems unattractive.

X activation by the binding of sequence-specific nonhistone proteins has the advantage of being rather easily generalized to the regulation of most genes during differentiation. However, I argue that these models have difficulty explaining the permanence of X chromosome differentiation. DNA replication should create a crisis, often leading to loss of differentiation. During replication, sequence-specific proteins are almost certainly "swept off" the DNA. It is reasonable to think that at least 100 base pairs are disturbed (strands separated; DNA:RNA hybrids; covered by polymerase, etc.) at each replication fork. DNA replication is a relatively slow process, proceeding at 30 to 50 nucleotides per second in mammalian cells (HUBERMAN and RIGGS, 1968; GAUTSCHI et al., 1973; HAND and TAMM, 1973). During 2 seconds, diffusion would cause a protein to become randomly distributed in the nucleus. A cluster of cooperative-binding proteins adjacent to one another along the DNA also would be destroyed as a replication fork moved through. Another difficulty is that new proteins would need to be made for each round of replication.

Occasionally a binding event should occur on the previously inactive X chromosome, leading to its activation. Of course, one can invoke late replication of the inactive X chromosome as essential for the maintenance of the differentiated state. However, in the mouse there is recent evidence that a change in the staining properties of one X may precede late replication by several days and replication events (TAKAGI, 1973). Furthermore, in some species, such as the shrew, the inactive X chromosome is not late replicating (RAO et al., 1970). Even in the adult mouse and rat, late replication may not be an invariant property of the inactive X (TIEPOLO et al., 1967).

There is another feature of the X inactivation phenomenon that I find particularly intriguing, and this is the evidence for "imprinting," a term introduced by CROUSE (1960) to describe changes in the state of a chromosome that occurs during one generation that allows it to be recognized as different in the next generation. In marsupials, X inactivation is not random, but rather the paternal X chromosome is always inactivated (COOPER et al., 1971; SHARMAN, 1971). It receives an "imprint" during passage through the male that allows it to be distinguished from the maternal X chromosome. The DNA of the paternal chromosome is not permanently altered, for the chromosome is active in the next generation. Integrated episomes (or the lack of integrated episomes) provide one reasonable model to explain imprinting. But are there alternate ways to change DNA permanently but reversibly?

I suggest that there is another reasonable model, involving DNA methyla-

tion, that can explain most aspects of X inactivation, including imprinting and has the additional advantage of providing an interesting model for gene regulation during differentiation. It will be seen that the known properties of bacterial DNA methylation enzymes are almost ideally suited for the proposed model. Thus, the evolution of the X inactivation system would be simple, requiring no new mechanisms. Because my model depends heavily on the remarkable properties of bacterial DNA modification (methylation) enzymes, a brief review of their properties will be presented first, followed by an argument that DNA methylation should have regulatory functions.

Bacterial restriction and modification enzymes

There have been several recent reviews of bacterial restriction and modification enzymes (ARBER, 1971; BOYER, 1971; MESELSON et al., 1972). Many elegant genetic and biochemical experiments have established the following: When foreign DNA (usually viral) enters *E. coli*, there begins a race between two events. The vast majority of the time a highly sequence-specific endonuclease (restriction nuclease) binds to a site on the DNA and cleaves the DNA, leading to the destruction of the foreign DNA molecule. Very rarely, the DNA is not cleaved, but instead the site in the DNA is methylated by a modification methylase. The sequence recognized by one restriction and modification system (*E. coli* RI) is shown in fig. 2. Many restriction and modification enzymes are now known, each specifically recognizing a different sequence (see SMITH and NATHANS, 1973, for a summary and suggested nomenclature). The product of the methylation event can be either N^6-methyladenine or 5-methylcytosine, depending on the methylase (fig. 3). For example, the *E. coli* RI and *E. coli* K modification methylases form N^6-methyladenine, whereas the *E. coli* RII enzyme forms 5-methylcytosine. As shown in fig. 2, the RI modification methylase is known to methylate two adenines symmetrically situated, one on each strand, inside the binding (or recognition) sequence (DUGAICZYK et al., 1974). *S*-adenosyl-L-methionine serves as the methyl donor for all known DNA methylases. Methylation of the recognition site completely protects the DNA from cleavage (MESELSON and YUAN, 1968). For the *E. coli* K host-controlled restriction and modification system, the endonuclease and the methylase are part of the same multimeric protein (HABERMAN et al., 1972). Both enzyme activities share a protein subunit thought to determine binding specificity.

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Fig. 2. The sequence of the recognition site for the *E. coli* R[DNA methylase and endonuclease, as determined by DUGAICZYK et al. (1974). The asterisks show which bases are methylated; arrows show where endonuclease cleavage occurs if the site is unmethylated.

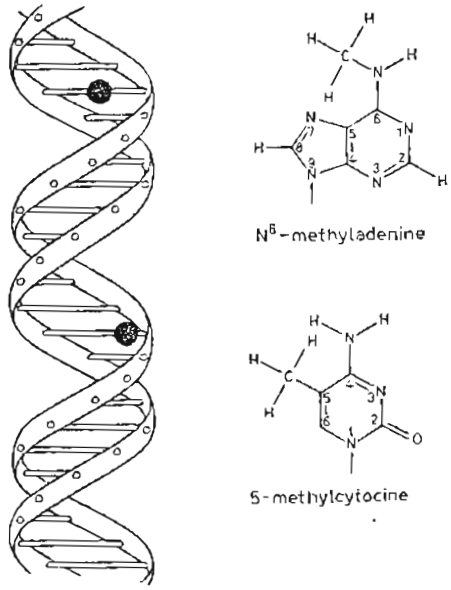

Fig. 3. Methylated bases in DNA. The location of the methyl group in the major groove of DNA is shown to the left of the chemical structure of the methylated bases.

The methylation of an unmethylated site is apparently a difficult task for the enzyme. The turnover time for the methylase activity on an unmethylated site has been estimated to be about 10 hours for the K enzyme (MESELSON and YUAN, 1972) or 7 hours for the B enzyme (LAUTENBERGER and LINN, 1972). Thus, methylation can be a very slow process. The genome of *E. coli* K contains many sequences (perhaps 1000) recognized by the K restriction endonuclease. Why doesn't the cell commit suicide every time its DNA is replicated? Suicide does not occur because a half-methylated site (one strand methylated) is fully protected from endonuclease activity (MESELSON

and YUAN, 1968; VOVIS et al., 1973), and the methylation of a half-methylated site occurs very rapidly, probably within 60 seconds, as estimated from in vivo work (BILLEN, 1968; LARK, 1968). The appropriate in vitro work has not yet been reported.

To summarize, there is evidence that the host-controlled DNA methylation enzymes of *E. coli* have the following properties: (1) highly specific recognition of base sequence in DNA; (2) very slow methylation of unmethylated sites; (3) fast methylation of half-methylated sites. Some of these and additional points will be considered in more detail later; in particular, I will consider how these properties can be used to establish and maintain a differentiated state, such as that seen for X inactivation.

Methylation should have a regulatory function

The substrate for the *E. coli* host-controlled modification methylase is double-stranded DNA having exactly the correct base sequence. The enzyme does not act on denatured DNA (see MESELSON et al., 1972). In these respects, the properties of restriction and modification methylases are very similar to those of the lac repressor, which recognizes base sequences in duplex DNA (GILBERT and MÜLLER-HILL, 1967; RIGGS et al., 1968, 1970a, b).

The products of methylation (5-methylcytosine or N^6-methyladenine) are capable of participating in normal Watson-Crick type base pairing and would not be expected to disrupt the DNA duplex, since the methyl groups are located in the major groove (see fig. 3).

I have argued that the lac repressor binds to the outside of duplex DNA and reads the edges of the bases exposed in the grooves (RIGGS et al., 1970a). Recent data on repressor binding to synthetic DNAs (RIGGS et al., 1972) and to isolated operator fragments (GILBERT, 1972; GILBERT and MAXAM, 1973) support this possibility; and ADLER et al. (1972) have proposed that the N-terminal region of the repressor fits into the major groove. Also, STEITZ and coworkers have obtained crystallographic data suggesting a model where the lac repressor binds to the outside of duplex DNA (STEITZ et al., 1974). A peptide fitting into the major groove might form hydrogen bonds using the N-6 hydrogen and the N-7 nitrogen of adenine. Methylation of adenine to form N^6-methyladenine causes three major changes: (1) a relatively large hydrophobic group is introduced in the middle of the major groove, (2) the N-6 hydrogen not needed for Watson-Crick base pairing is eliminated, and (3) there is steric hindrance between the methyl group and

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... N-7, probably eliminating the H-bonding capability of N-7. Thus, the formation of N^6-methyladenine *radically* alters the major groove and ought to affect regulatory protein binding. 5-Methylcytosine also should effect regulatory protein binding because it is well established that the lac repressor is very sensitive to minor changes in the major groove, such as those caused by the substitution of bromodeoxyuridine for thymidine (LIN and RIGGS, 1970b, 1972). Therefore, it is reasonable to think that methylation will change the affinity of DNA-binding proteins for their binding sites. The argument is even stronger because it is well established that the *E. coli* K restriction and modification enzyme has a strong, easily detectable affinity for an unmethylated site but no detectable affinity for a methylated site (YUAN and MESELSON, 1970). Therefore, *methylation is known to affect the binding of sequence-specific proteins to DNA.* It is interesting to note that most methylations need not be regulatory, but those methylations that occur in operator sites are very likely to affect regulatory protein binding.

A DNA methylation model for X inactivation

First, I will outline the model as it applies to eutherian mammals and then consider each of the essential points in more detail. As illustrated in figs. 1 and 4, I propose that in the zygote, before the differentiation of the X chromosome, the primary inactivation center is not methylated. The methylation of an unmethylated inactivation center is proposed to be a slow process, requiring, on the average, many hours. However, eventually one of the primary inactivation centers is methylated. This results in the activation of that chromosome (perhaps the methylase no longer binds and acts as a repressor). As a direct or indirect result of this activation event, two proteins are synthesized (or one protein with two functions). One protein functions to change the methylase so that the methylation of an unmethylated inactivation center becomes impossible (it was very slow to begin with). The altered methylase still can methylate quickly a half-methylated inactivation center that has one strand methylated. A second protein functions to condense and inactivate any X chromosome with an unmethylated inactivation center. As shown in fig. 4, given a methylase capable of methylating a half-methylated inactivation center, but unable to methylate an unmethylated inactivation center, the differentiation of the X chromosomes will be maintained easily through successive cell divisions and DNA replication events. Now I will consider some of the essential postulates in more detail.

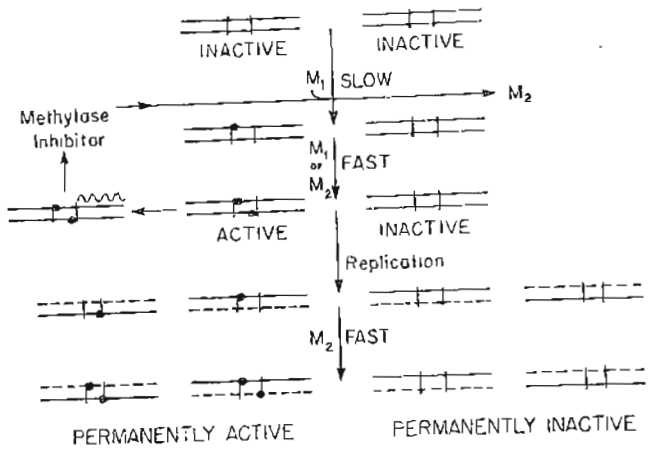
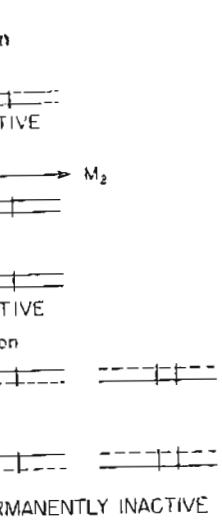


Fig. 4. A DNA methylation model for X inactivation. Given a sequence-specific DNA methylase that acts efficiently only on half-methylated sites, the differentiated state of the X inactivation center can easily be maintained through DNA replication. See text for additional details.

1. One primary X inactivation center. This is in keeping with most data and other models, and seems essential to explain the randomness of inactivation and the O^{hv} mutation (see below). It is convenient to have some symmetry in the base sequence of this site. That is, a sequence of bases on one strand in the left half of the inactivation center is repeated in reverse order on the other strand in the right half of the binding site. Such symmetry insures that the two half-methylated sites produced by replication of a fully methylated site are identical with regard to recognition by the methylase. Such symmetry in the recognition sites for bacterial modification methylases is well established (see fig. 2).

2. Sequence-specific DNA methylase. The most important postulate is that an autosomally located DNA methylase exists that (a) is highly sequence specific, (b) methylates the inactivation center symmetrically on both strands, and (c) methylates an unmethylated inactivation center with great difficulty but readily methylates a hybrid center (one strand methylated). As already pointed out, these are properties in keeping with present data on the *E. coli* host-controlled DNA methylases.

3. Methylase inhibitor. In order to explain that only one X chromosome is activated *per chromosomal set*, it is convenient to postulate an X-linked function. I propose that the X chromosome codes for a protein that interacts



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with the methylase (M_1, see figs. 1 and 4) and slightly inhibits its activity so that methylation of an unmethylated inactivation center no longer occurs to any significant extent. However, it is proposed that the altered methylase (M_2) still can quickly methylate a hybrid, half-methylated site. There is no need to postulate a small number of methylases; it is only necessary to have the methylase inhibitor produced at the same or slightly higher level than the methylase. If the methylase and methylase inhibitor bind stoichiometrically, this simple feedback loop results in one active X per diploid autosomal set.

The model proposed here seems to account for the following aspects of X inactivation: *Permanence.* Once established, the differentiated state of the X inactivation center would be maintained. *Random inactivation in eutherian mammals.* Randomness follows easily if the critical methylation event takes place after fertilization and if the X inactivation center is unmethylated in the sperm. *Paternal inactivation in marsupials.* Preferential maternal activation would result if methylation of the inactivation center occurred before fertilization. Note that, in general, "imprinting" can be explained by the state of methylation of DNA in the egg and sperm. *The* O^{hv} *mutation.* Recently a mutation (O^{hv}) was found, mapping in or near the inactivation center of the mouse X chromosome, that results in preferential activation of the X carrying the mutation (DREWS et al., 1974). This mutation may increase the affinity of the inactivation center for the methylase or increase the probability of methylation. *Aneuploids and polyploids.* These are adequately accounted for by the simple feedback loop described above. The level of X-linked methylase inhibitor is automatically adjusted to match the number of sets of autosomal methylase genes. For example, in tetraploids, methylation would continue until two X chromosomes were activated. *Reversibility for the next generation.* The differentiated state resulting from methylation during developmental history is erased whenever DNA methylation is prevented during DNA replication. This may occur during oogenesis or spermatogenesis or during the rapid DNA replication cycles following fertilization.

The model presented above does *not* explain the totality of inactivation, i.e., the mechanism whereby inactivation and condensation spreads from the inactivation center to encompass the entire X chromosome. However, the mechanism for the spread of inactivation may well be entirely different from that for the initiation and maintenance of the differentiated state of the inactivation center. Thus, the mechanism of the spreading effect is not a major consideration of this paper.

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Methylation and differentiation

The general idea that DNA methylation might have a regulatory role is not new. Many authors have suggested such a role (SCARANO et al., 1967; SNEIDER and POTTER, 1969; ADAMS, 1970; MAMELAK and BOYER, 1970; VANYUSHIN et al., 1970; HARRISSON, 1971; COMINGS, 1972). However, to my knowledge, eukaryotic methylation has not been discussed in light of the recent evidence accumulating about the *E. coli* lac repressor and DNA methylases, nor has emphasis been placed on the advantages of methylation for permanent changes in regulation occurring during differentiation. An important exception is that SCARANO has proposed a model for differentiation based on DNA methylation (SCARANO et al., 1967; SCARANO, 1971; TOSI et al., 1972). He suggests that 5-methylcytosine produced by DNA methylases might be deaminated at the DNA level to produce thymine. One round of replication would lead to a transition from a G-C base pair to an A-T base pair. Thus, methylation would result in directed mutation, an event that, of course, would have permanent regulatory consequences when occurring in operators. This is an interesting model, but at present there is no firm evidence for the deamination of 5-methylcytosine to thymine at the DNA level (see SNEIDER, 1973).

The major point to be made here is that the properties of restriction and modification methylases are almost ideally suited to the establishment of stable differentiated states, without the necessity of proposing mutational events. Since the methylation of an unmethylated site is quite difficult, it is reasonable to think that very stringent conditions must be met for the initial methylation event to occur. However, once a site is methylated, it remains methylated because the methylation of a half-methylated site is easy and probably not sensitive to minor environmental fluctuation. Thus, "irreversible" differentiation is easily programmed; stable circuits are easily constructed. I suggest that the reader see COOK (1974) and substitute the words DNA methylation for "superstructure."

Present and future experiments in eukaryotes

Many of the points emphasized in this paper are, at least in principle, experimentally testable. A search of the literature showed that a number of interesting experiments already have been done. The results of some of these experiments will be summarized here along with suggestions for future work

Methylation should be regulatory and lead to the activation of genes. DNA isolated from the genetically active macronucleus of *Tetrahymena* contains N^6-methyladenine, whereas DNA from the inactive micronucleus contains at least 10-fold lower levels of this methylated base (GOROVSKY et al., 1973). Additional experiments comparing active and inactive DNA are of obvious interest. It would be particularly interesting to look at the content of methylated bases in the inactive set of chromosomes in the mealybug (see CHANDRA, 1971). PARSA et al. (1973) have reported recently that embryonic rudiments of rat pancreas fail to differentiate in a methionine-deficient medium, even though growth is not affected. Their studies also suggest that it is not a deficiency of methionine per se that blocks differentiation but rather a deficiency of *S*-adenosyl-L-methionine, which is the methyl donor for DNA methylase.

Sequence-specific DNA methylases should exist. A cytosine methylase has been studied in mammalian cells (KALOUSEK and MORRIS, 1969; DRAHOVSKY and MORRIS, 1971a, b), but this enzyme methylates single-stranded DNA and does not seem to have properties desirable for the establishment and maintenance of differentiated states. 5-Methylcytosine is the only methylated base yet found in mammalian DNA (VANYUSHIN et al., 1970; LAWLEY et al., 1972), and its level is fairly high, 2–4% of total DNA cytosine. Because of the high level of methylation, it seems likely that most DNA methylation is not involved in specific gene regulation. However, the content of 5-methylcytosine may be tissue specific in the organs of the developing chick embryo (KAPPLER, 1971). The high background of 5-methylcytosine and the presence of the cytosine methylase studied by DRAHOVSKY and MORRIS (1971a, b) will make it difficult to search for additional DNA methylases producing 5-methylcytosine. Fortunately, the level of N^6-methyladenine in mammalian cells is very low (VANYUSHIN, 1970; LAWLEY et al., 1972), and this may aid in future searches for methylases methylating only certain operators, promoters, chromosomes, or the X inactivation center.

The DNA of egg, sperm, and the inactive X chromosome should be undermethylated. It was interesting to find that sperm DNA is, indeed, 50% undermethylated when compared to other tissues of the same species (VANYUSHIN, et al., 1970). My model requires that at least the inactivation center of the inactive X chromosome be unmethylated. Of course, it will be very difficult to measure the methylation of just this site. However, there is already evidence in the literature suggesting that the entire late-replicating X chromosome may be undermethylated. The inactive X chromosome is late replicating and probably comprises a sizable percentage of the late-

replicating DNA in most species. In cells of the Chinese hamster and mouse, it has been reported that late replicating DNA is 20–50% undermethylated (ADAMS, 1971; COMINGS, 1972).

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