

DNA Modification Mechanisms and Gene Activity during Development

Developmental clocks may depend on the enzymic modification of specific bases in repeated DNA sequences.

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It is generally accepted that the differentiated state of a given type of cell is associated with the activity of a particular set of genes, together with the total inactivity of those sets associated with the differentiation of other cell types. It is also clear that the differentiated state of dividing or nondividing cells is often extremely stable. In this article we suggest mechanisms that may account for this stability and that also attempt to explain the ordered switching on or off of genes during development.

The phenotype of the organism depends on the genotype, and the genetic contribution from both parents is in almost all cases equal. Since the ultimate control of development resides in the genetic material, the actual program must be written in base sequences in the DNA. It is also clear that cytoplasmic components can have a powerful or overriding influence on genomic activity in particular cells, yet these cytoplasmic components are, of course, usually derived from the activity of genes at some earlier stage in development. A continual interaction between cytoplasmic enzymes and DNA sequences is an essential part of the model to be presented.

Modification Enzymes

In bacteria, enzymes exist which modify DNA by methylating adenine in the 6-position (1). These enzymes are extremely specific in their action; they modify bases at particular positions in short defined sequences of DNA, which, at least in some instances, form a palindrome. (A palindrome in DNA is an inverted duplication, with twofold rotational symmetry. The 3' → 5' base sequence is the same on each strand.) These modifications prevent the DNA

being degraded by restriction enzymes, which are equally specific in their action. In higher organisms, bases are also modified: 5-methylcytosine is a common component of DNA (2), and 6-methyladenine has been identified in simple eukaryotes (3). It is not yet known whether these modifications occur at specific sites. In the case of transfer RNA (tRNA) of both bacteria and higher organisms, a number of bases are modified at specific sites (4).

The methylation of adenine in DNA is not heritable in the usual sense, but a bacterium with a modification enzyme could, in principle, have a very different phenotype to one without if the presence or absence of methylation affected transcription. Hawthorne (5) and Scarano (6) have suggested that certain other base modifications could lead to heritable changes in base sequences and that these could control the activity of adjacent structural genes. We explore these possibilities further and suggest that such changes could operate developmental clocks which turn genes on or off after a specific number of cell divisions. In addition, we propose that the same ordered control of the transcription of genes could be achieved by the methylation of bases, without changes in sequence. The modification mechanisms are as follows.

1) To explain the instability of the mating type loci in certain strains of yeast, Hawthorne (5) has suggested that an operator region could exist in two alternative states. One state has A · T (adenine · thymine) base pairs at particular sites in the controlling region, and the other has G · C (guanine · cytosine) base pairs at the same sites. The transition from A · T to G · C or G · C to A · T requires cell division, and it occurs as follows. The modification of adenine at particular sites could occur

by the removal of an amino group at the 6-position. This gives rise to inosine (I), and it is known that inosine base pairs with cytosine rather than thymine (7). Therefore one round of replication after modification will produce A · T and I · C, and at the following replication a G · C base pair is formed. The reverse transition occurs by the action of a second modifying enzyme, which removes an amino group from the 6-position of cytosine to give uracil (U). After two rounds of replication, the original A · T base pair is restored. These transitions are illustrated in Fig. 1a. The change in base sequence that occurs is irreversible if only one of the two modifying enzymes is present. Since there is now genetic evidence that mismatched bases in DNA are repaired to give normal base pairing (8), Hawthorne suggests that the modification occurs in the short stretches of single-stranded DNA in the replication fork. There would therefore be no opportunity for the repair of mismatched bases such as I · T or G · U.

2) Another possibility, which has been discussed by Scarano (6), in connection with the problem of differentiation, depends on the methylation of cytosine at the 5-position, followed by deamination at the 6-position to give thymine. In this way a G · C pair would be changed to an A · T pair after replication (Fig. 1b). The amination of thymine to 5-methylcytosine, which pairs with G, will give the reverse change.

3) To maintain methylated bases in DNA, a modification enzyme must always be present. To segregate methylated from nonmethylated cells, two enzymes are necessary. One model is illustrated in Fig. 2. The first enzyme, E1, methylates one strand within a stretch of palindromic DNA and the other strand just outside this stretch. This does not provide a substrate for the second enzyme, E2, until replication occurs, and then only one of the daughter molecules is methylated by E2. This enzyme resembles bacterial modification enzymes in adding a methyl group to the other half of the palindrome, but it differs in not acting on nonmethylated DNA. [The same discrimination between half-methylated and nonmethylated DNA is shown by restriction enzymes of bacteria (1).] Therefore, once both strands of the se-

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quence are methylated, all subsequent progeny are modified, provided that E2 remains present. The other product of the first division segregates further methylated cells as long as E1 is present, but in its absence only nonmethylated progeny will be formed. The methylated state could be extremely stable, as the methyl groups would be diluted out only if the modification enzyme is lost through mutation in its structural gene. However, an essential part of our model is the switching on or off of the genes for modification enzymes during development or differentiation.

In bacteria and their viruses, specific mechanisms for the control of gene activity at the level of transcription are well known, and it has been shown that operator regions have palindromic features (9). It is generally believed that similar control mechanisms must exist in higher organisms. There are several simple ways in which changes in base sequence or methylation could determine whether or not a particular gene is transcribed, some of which have already been discussed by Venner and Reinert (10). One possibility is that the sequence where modification occurs is also an operator sequence to which a repressor binds. In one state of the DNA the repressor binds to the operator and the contiguous structural gene is inactive. In the other state the repressor does not bind to the operator and transcription occurs. Alternatively, modification could occur in the promoter sequence, that is, the short region of DNA to which the transcribing RNA polymerase first binds; in one modified state the gene would be transcribed and in the other it would not. [It is known in several instances that promoter regions contain short palindromes, since they can be attacked by restriction enzymes (11).] Binding sites for RNA polymerase will be common to many or all structural genes, yet the modification enzyme is specifically inactivating or activating particular genes. We must therefore postulate that the specificity of binding is provided by a defined sequence adjacent to the promoter, but that modification actually occurs in the promoter region. A third possibility, even simpler, is that base changes would simply introduce (or remove) a polypeptide chain terminating sequence within a structural gene.

In the subsequent discussion we often refer to enzymes which modify DNA as controlling enzymes and to their substrates as controlling sequences.

Somatic Segregation of Gene Activities

The modifications outlined in Figs. 1 and 2 can result in the formation of an unaltered cell, together with one in which a particular gene is activated or inactivated. This situation is like that of a stem line cell which continually divides to form cells with new functions. The stem line cell is unstable, but the daughter cells which are modified are quite stable. However, although the switching on of a single gene may commit the cell to differentiation, it is unlikely to be sufficient to bring about all the changes required for differentiation. One obvious possibility is that the first activated gene codes for another modifying enzyme that is active at several other sites in the genome, which have the same controlling sequence. This may, for instance, shut off genes whose activity is necessary for cell division and

turn on others which synthesize those proteins that give the cell its particular properties. It is easy to see how somatic segregation could lead to the triggering of sequential regulatory events, or the type of cascade regulation discussed by Britten and Davidson (12) and Pontecorvo (13). A further possibility is where a gene is switched on transiently. A controlling enzyme may modify the controlling sequence adjacent to its own structural gene. The enzyme is first switched on by the action of some other controlling enzyme, but as soon as it is synthesized it overrides the action of the first enzyme by reversing the modification. In this way a controlling enzyme would only be present for one or two cell divisions, but during this time it could, of course, affect the activity of other genes.

Certain complications could arise when one considers the possible segre-

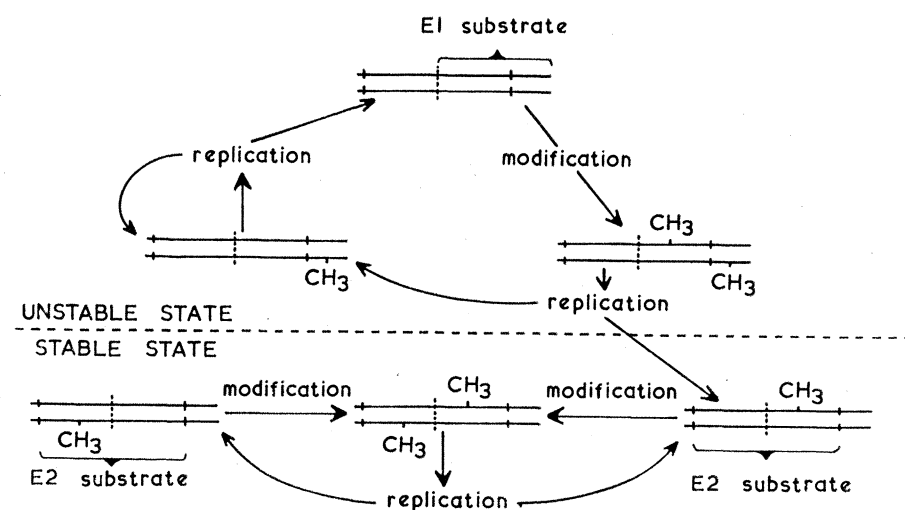
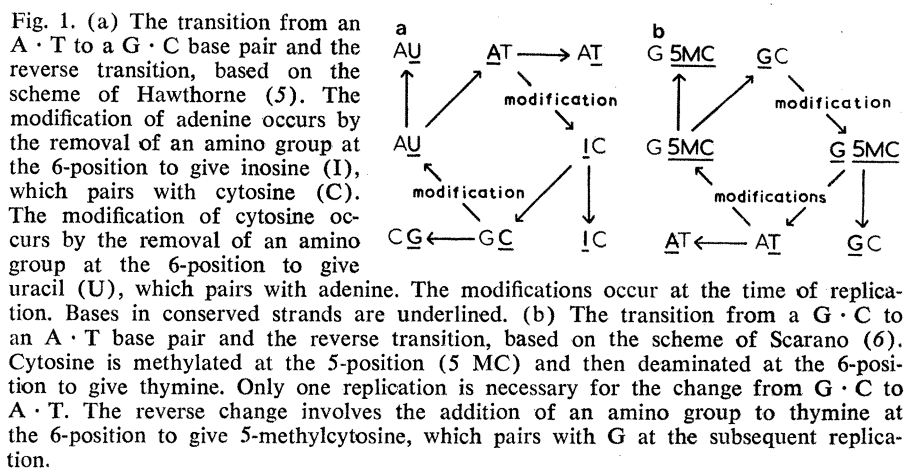


Fig. 2. The segregation of methylated DNA from an unmethylated precursor. The first modification enzyme, E1, methylates one-half of a palindromic sequence and an adjacent sequence in the complementary strand. Replication provides one substrate for the second enzyme, E2, which methylates the other half of the palindrome and all subsequent progeny molecules. In the presence of E1 and E2, unmodified or partially modified cells continually give rise to stable modified ones. If E1 is inactivated or disappears, stable modified and unmodified cells are formed.

gation events that can occur in the division of a diploid cell. Each controlling sequence occurs on each homolog, and therefore modification at both sites will result in the production of daughters with one, two, or no modifications in the ratio 1 : 2 : 1. In many instances this may not matter, as a single activated gene may set in train all the required changes. Another possibility which we favor is that controlling sequences are modified and activate genes on only one of the two homologous chromosomes. In differentiated antibody forming cells, only one of the two structural genes in a heterozygote is transcribed (14), and in female mammals only one of the two X chromosomes is active (15). The mechanisms discussed suggest how modification could occur in only one of two homologous chromosomes in a cell. Suppose the controlling sequence is a

substrate for both a repressor and a modification enzyme, E1, but the affinity of the repressor is very much greater. There is therefore a low probability of modification, and a very much lower probability that both controlling sequences on both chromosomes will be modified in one cell generation. Once modification has occurred, it prevents repressor binding and allows transcription of the adjacent structural genes. The products of these inactivate E1 and switch off its synthesis. Provided that the initial modification is in both arms of a palindromic sequence, a maintenance enzyme, E2, keeps one chromosome methylated in all subsequent generations, as in the lower half of Fig. 2, whereas its homolog is unmethylated and remains so. We do not propose that this simple model will alone account for whole X chromosome inactivation.

Segregation of gene activities is not the only important event, as we have to consider also the mechanisms whereby all the progeny from a particular cell are altered or differentiated in the same way at a particular time in development. The application of the model to this situation is developed below.

Developmental Clocks

It can be readily seen how in principle the modification mechanism could enable a cell to count the number of divisions it has gone through during a particular stage in development. Consider the hypothetical repeating sequences shown in Fig. 3a. At the right end there is a sequence to which the modification enzyme binds. This sequence is first modified by an $A \rightarrow I \rightarrow G$ transition. When this has occurred, the site of action for the enzyme has now moved eight bases to the left. This process will be repeated as many times as there are repeats of the sequence. At the end of the precisely determined number of divisions, the operator or promoter site is altered in the way that has been mentioned and the developmental switch comes into operation.

Such a developmental clock will not operate precisely if the bases modified are on one strand. In this case modified and unmodified strands segregate, and the subsequent progeny of a single cell will have modified a varying number of control sequences. This difficulty is avoided if both strands are modified (Fig. 3b). There is a binding sequence for the controlling enzyme which can exist in two forms, differing in at least two base pairs. It is adjacent to a very similar sequence which will be modified in both strands by the enzyme. These sequences when modified become the same as the binding sequences. Therefore the modifications move progressively from one end of the region of repetitive DNA to the other, and the divisions are counted.

Methylating enzymes may also count cell divisions. Of several possible mechanisms we describe one (Fig. 4). The clock is started by E1, which acts on a specific substrate at one end of the repeated sequences. It methylates one strand, and this is an essential signal for the second enzyme, E2, which inserts further methyl groups on both strands within the next sequence. This enzyme cannot act on DNA methylated on both strands in one sequence, but it does so

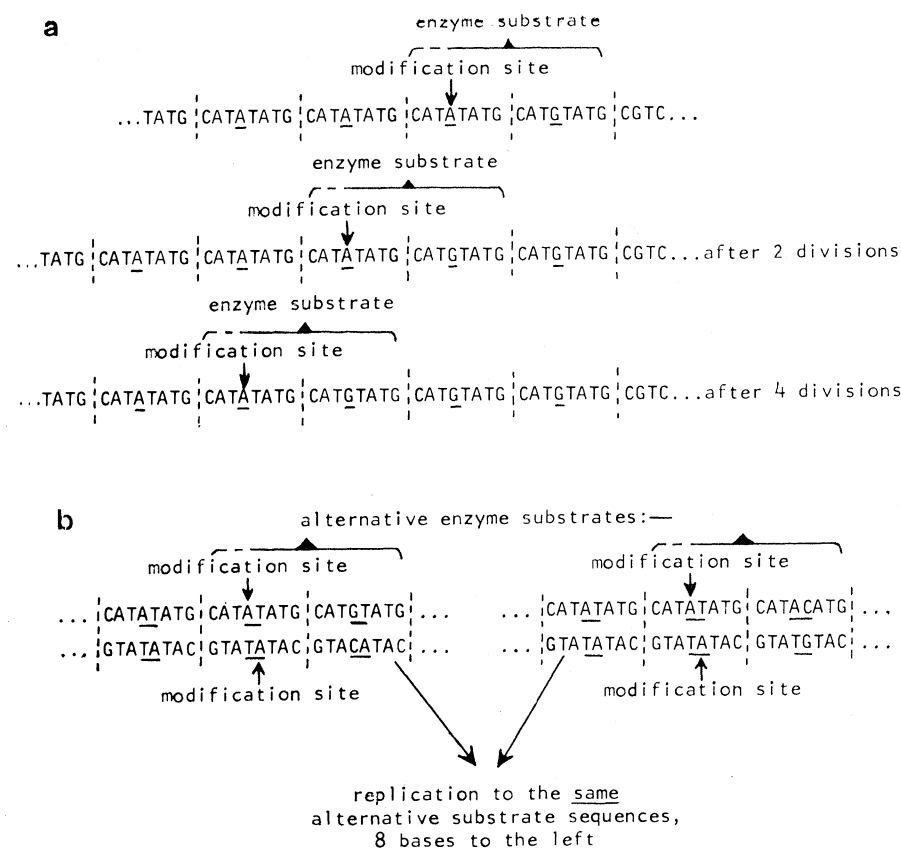


Fig. 3. (a) A mechanism for counting cell divisions based on the $A \cdot T \rightarrow G \cdot C$ transition in Fig. 1a. The modification enzyme recognizes the first sequence of eight bases, because it contains G at the 5-position, together with the whole or some part of the sequence to its left. The A at the 5-position of this second sequence is changed to G to give a new recognition sequence eight bases to the left. When all the sequences have been successively modified, a structural gene at the extreme left of the repeated controlling sequences (not shown here) is activated. (b) The modification of both strands of a controlling sequence. The controlling enzyme recognizes a sequence which has a G · C pair at either the 4- or 5-position. (If each controlling sequence forms a short palindrome, the recognition sequences can be structurally identical, as shown here.) In both cases it modifies A at the 4- and 5-position of the sequence to the left. Both these modified sequences then become recognition sequences after replication. The modifications therefore move progressively from right to left and count cell divisions as in (a).

after replication again provides substrates with only one methylated strand. In this way an additional segment of the clock is modified at every division until the end is reached, whether or not all the sequences behind the growing points are methylated.

In both these types of clock, all the offspring from a progenitor cell will reach the same stage of development after they have gone through some specified number of divisions. The clock may, of course, trigger one or several segregation events that lead to specific differences in cell types within the clone. Separate clocks could run sequentially at the same time, overlapping each other in time within one cell lineage.

Britten and Davidson (12) have pointed out that the existence of multiple repeats of DNA sequences in the genome suggests that common regulatory sequences may be adjacent to many different structural genes. The developmental clocks that we have described would suggest an additional function for repetitive DNA which is not transcribed. These sequences would be tandem repeats of palindromes. Evidence for the existence of many such sequences in the DNA of higher organisms has been obtained (16).

Development of the Chick Limb Bud

The recent experiments on the early development of the chick wing (17) provide a convincing example of a developmental clock. The tip of the limb bud, which is called the progress zone, contains dividing cells, and the products of division form in strict sequence the various structures of the limb from its base to the extremity. If the progress zone from a limb in which the basic structures are nearly fully formed is transplanted to a very young limb from which the progress zone has been removed, then none of the structures are produced. On the other hand, if a young progress zone replaces one on the end of a wing which has already laid down all essential structures, then another wing is formed at the end of the first. In this case, the sequence of bone rudiments would be humerus, radius or ulna, hand, humerus, radius or ulna, hand. These results show that there is a temporal order in the laying down of successive structures, and this order might very well be related to the number of cell divisions that have elapsed in the cells of the progress zone.

A Clock for Aging?

The life-span of an organism is under genetic control, and it has frequently been asserted that there must be a developmental program for aging. More specifically, it has been suggested that the aging program might be related to division potential of cells, because diploid cells in culture have a clearly defined life-span which is dependent on the number of population doublings rather than chronological time (18). Current interest in mechanisms of aging has centered around error theories, for which some evidence has been published. If, instead, the life-span of these cells is programed, we think that a clock of the type outlined in Figs. 3 and 4 might provide the necessary specificity in doubling potential before senescence and cell death occurred. When the clock runs out, there are many possible deleterious or lethal events that might be triggered. For instance, the enzymes for chromosome replication or any other essential cellular function may be switched off; alternatively, there may be a general reduction in the ac-

curacy of information transfer between macromolecules.

There is no doubt that programed death of certain tissues or groups of cells is a normal component of embryogenesis and development (19). This program could be based on the clock mechanisms we suggest; furthermore if restriction enzymes (specific deoxyribonucleases) (1) occur in higher organisms, substrates for these might be created by the loss or gain of modification enzymes in particular cells, and this would be followed by the degradation of the DNA and death of these cells.

The Developmental Program

The combination of developmental clocks and precise segregation mechanisms which together determine which genes will be activated provides the essential requirement for an ordered genetic program for development. One can describe the determined changes as being part of a developmental tree, where, at precise times during development, cells branch out into different

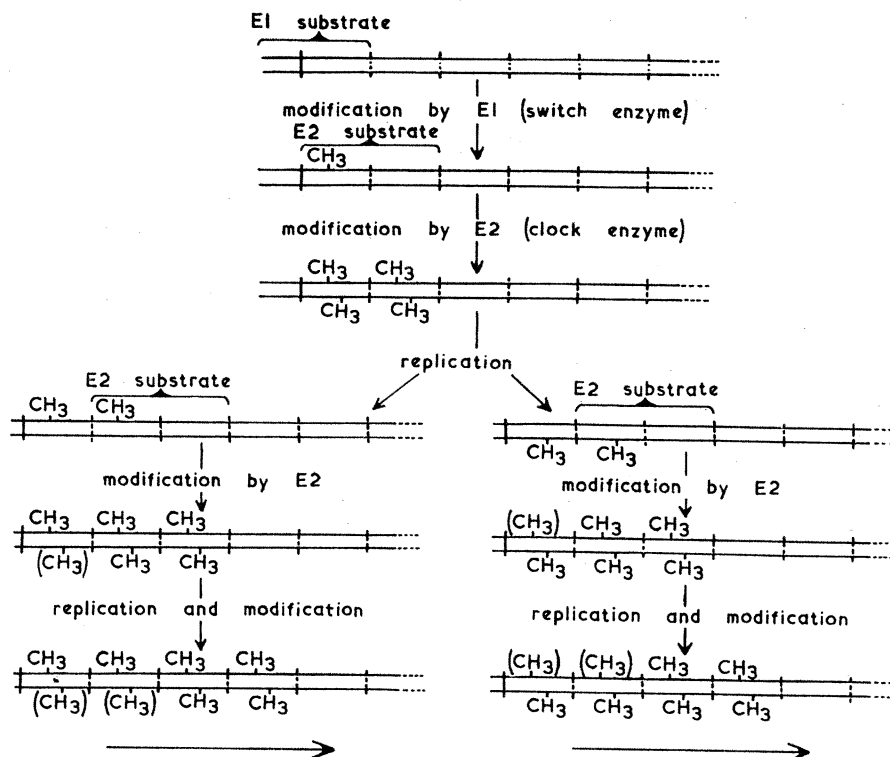


Fig. 4. A mechanism for counting cell divisions based on the methylation of palindromic controlling sequences. The first enzyme, E1, switches on the clock by recognizing a starter sequence, at the extreme left, which is adjacent to the first of the repeated sequences of the clock. One strand of this sequence is methylated by E1, and this provides a substrate for E2, which inserts three more methyl groups in the first two controlling sequences. E2 does not act further once both strands are modified. However, after replication new substrates of E2 are formed, allowing the next sequence to be methylated. (All the sequences behind the "growing point" may become modified, but this does not affect the clock mechanism.)

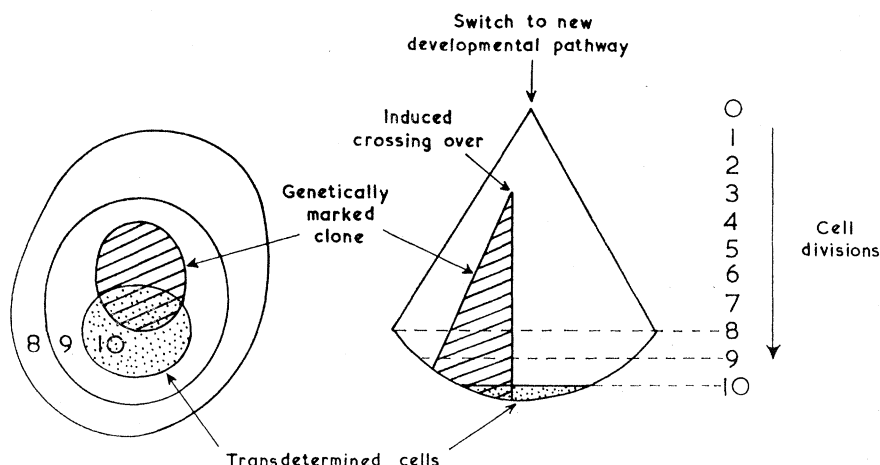


Fig. 5. An explanation for transdetermination based on an event in a single cell. The diagram on the right represents a clone derived from a cell in which a developmental clock has been triggered. It takes ten divisions for the clock to induce transdetermination in a group of cells. The induction of mitotic crossing-over by irradiation occurs after the clock has started and it produces a genetically marked clone (or patch of cells, as shown on the left) which can overlap the transdetermined region (33).

sublines that later themselves become subdivided into more diverse classes. At each stage the cells become more and more tied to a specific pathway of development and more and more distinct from cells derived from branches of the tree. Moreover, once a differentiated state has been reached, the model explains why it is so stable. Base changes are obviously stable, and methylated bases are maintained provided that the necessary enzyme is present. They would be lost only as a result of mutation in the structural gene for the modification enzyme.

Although the model suggests that development is clonal, it should be noticed that specific events can occur in groups of cells rather than individual ones. For instance, after fertilization a developmental clock or clocks may be set so that after n divisions one or more segregation events are triggered. At this time the 2^n cells that have been formed may segregate into two or more types of cell. Embryonic cells with specific cell surfaces can recognize each other (20); these cell types may therefore aggregate together into groups. This is possibly what happens when different embryos are fused to form mosaic allophenic mice (21).

The model described is more likely to provide an explanation for the ordered development of embryos of the mosaic type than it is for those of the regulative type. In the former, exemplified by *Drosophila* (22), the parts of the embryo are rigidly determined to develop into particular larval or adult structures. Removal of groups of cells of the deter-

mined embryo results in loss of specific differentiated structures. In the latter, removal of parts of the developing organism, or the inhibition of cell division, may simply result in the formation of a smaller complete organism at some later stage in development (23).

We do not wish to underestimate the importance of a cell's environment in the determination of its subsequent fate during development. It is, for instance, widely believed that the pattern of development is determined by fields or gradients set up by organizing or signaling cells or groups of cells (24). We would simply point out that the origin of such a situation must initially depend on programmed differences between cells in the developing organism, and some of these differences could come about in the way suggested. Moreover, some of the switches or clocks we have discussed could be triggered by hormonal or other influences, or alternatively they may determine how a cell will respond to such stimuli. It may be significant that some cells can accept positional information only if they are first appropriately conditioned by cell division (25).

Totipotent Nuclei

In extensive experiments by Gurdon and his associates (26), nuclei from differentiated cells were injected into anucleate eggs. For instance, nuclei from tadpole intestinal epithelial cells supported embryonic development to a stage where many types of differentiated

cell were present, and in some instances adult animals were formed. In other experiments, nuclei derived from adult skin tissue were successfully transplanted into anucleate eggs. These results show that nuclei in differentiated cells can be reprogrammed by egg cytoplasm: they are totipotent because they can subsequently give rise to all other types of cell. There are, however, types of differentiated cell such as neurons, which contain nuclei that do not support normal development after transplantation, and in these cases the changes in genetic activity that occur during differentiation may be irreversible.

The modification mechanisms described are all reversible; therefore it is possible that a battery of specific enzymes exists in the egg cytoplasm which recognize controlling sequences and reverse many—although not necessarily all—modifications. The specificity of these enzymes may be somewhat less than those which originally introduced the modifications during development. For instance, all the modified bases in the repeated sequences of a developmental clock could be erased at a single step. It is not at all unlikely that there is a special mechanism for reprogramming in the egg cytoplasm, since apart from the transplantation experiments just mentioned, the oocyte and the spermatozoan are highly specialized products of meiotic division, the nuclei of which themselves have to be reprogrammed. Nevertheless, we find it hard to believe that reprogramming could actually involve base changes in the DNA, and for this reason we tend to prefer the version of our model that depends on methylation of particular bases. A general demethylating enzyme is a possibility (provided that it was removed before the embryo started development), but we prefer the following alternative.

If the egg cytoplasm contains no maintenance enzyme, then methyl groups will simply be diluted out during the early cleavage divisions. At each nuclear division the number of chromosomes containing methyl groups will be reduced by half. After x divisions the probability of any one chromosome remaining modified is $2n/2^x$, where n is the haploid chromosome number. Where n is between 10 and 30, it would need between 11 and 13 divisions to reduce the number of cells containing at least one modified chromosome to 1 percent. We suggest that an initial clock, immune from the diluting out process,

would set in train the whole process of development after approximately this number of nuclear divisions has occurred.

Effect of Bromodeoxyuridine on Differentiation

Perhaps the strongest evidence that DNA is directly implicated in differentiation comes from numerous studies with the thymidine analog bromodeoxyuridine (BrdU). It has frequently been shown that low concentrations of BrdU which are nontoxic to cells specifically inhibit differentiation or development. There is no effect if excess thymidine is added at the same time as the analog, but in several instances once the BrdU is incorporated into DNA, the block in differentiation cannot be reversed by adding excess thymidine. Only a few of the many examples of the specific action of BrdU can be mentioned [for a full review, see (27)]. Myogenic cells can be cultivated in vitro for several days. After this time, DNA synthesis ceases, the cells fuse to form multinucleate tubules and synthesize the contractile proteins actin and myosin. Bromodeoxyuridine does not prevent the myoblasts from proliferating, but its presence, even for one cell division, completely inhibits their differentiation (28). Mesoderm of the chick limb bud differentiates into cartilage in cell culture, and this differentiation is irreversibly blocked by the substitution of approximately 2 percent of the thymine in DNA with the bromo analog. After treatment with BrdU is terminated, the analog rapidly disappears from the dividing cells, but even so differentiation does not then occur (29). In other instances, the analog is diluted out by replication and differentiation follows. Finally, it has been shown that BrdU blocks the development of embryos if applied at an early cleavage stage (30).

We propose that these effects are brought about by the substitution of a bromine atom for the methyl group on the 5-position of thymine, and this prevents the normal modification of controlling sequences during development. This could occur either by preventing the loss or gain of a methyl group of a particular pyrimidine base (for instance, in the change from cytosine to thymine previously mentioned) or, more generally, by altering the action of modification enzymes on controlling sequences containing BrdU-substituted DNA.

Determination and

Transdetermination in *Drosophila*

The stability of differentiated cells has already been mentioned, but we now turn to the remarkable studies of Hadorn and his associates (22, 31), who have demonstrated that the determined state of undifferentiated larval cells can be very stable. The adult structures of *Drosophila* are formed during metamorphosis from imaginal discs in the larva. Imaginal disc tissue can be grown in the abdomen of adult flies and continuously propagated by transfer of pieces of tissue to fresh adults. Disc tissue reimplanted in larvae differentiates during metamorphosis to produce a particular adult structure, such as part of a wing, leg, or antenna. This is triggered by the hormone ecdysone, which activates the developmental program and allows the further events required for differentiation to proceed. A particular line of disc cells is determined to produce a specific adult structure. This is inherited from cell to cell, as disc fragments have been subcultured for more than 70 transfer generations over a period of several years without any change in determined state. We suggest that this stability is due to the inheritance of appropriate modifications in their DNA. Sometimes disc tissue that is determined to develop in one direction spontaneously changes to another determined state. This transdetermination never occurs in the absence of proliferation; indeed, its frequency is related to the number of cell divisions which have occurred. If during growth the modifications are occasionally lost, then these cells may move into a determined state distinct from the first one. It is a characteristic of transdetermination that specific changes occur more frequently than others and that successive changes follow particular pathways.

Kauffman (32) has presented a detailed model for determination based on the setting of a number of bistable states, or developmental switches. The various pathways for transdetermination are explicable if the setting of the switches alters with given frequencies, one state changing to the other more frequently than the reverse change. His analysis is quite consistent with our model if the switches are modified or unmodified states of particular controlling sequences and one change, for instance the failure to methylate DNA, is more frequent than the reverse.

By use of mitotic crossing-over to

mark particular groups of cells, it is possible to show that a patch of tissue in which transdetermination has occurred can occasionally overlap one which has arisen as a result of mitotic crossing-over in a single cell. It is therefore impossible for each patch to be an individual clone, and it has consequently been argued that transdetermination occurs in groups of cells (33). However, this argument no longer holds if transdetermination depends on two events: first, the reversion in one cell to a predetermined state, then a given amount of proliferation to a new determined state. If only a proportion of the cells in the clone have undergone sufficient divisions to reach the new determined state (and such cells are known to aggregate together), then it is quite possible for the patch from mitotic crossing-over to be included within this larger clone, only part of which has undergone transdetermination (Fig. 5).

Homoeotic mutants are those that produce developmental defects analogous to transdetermination. For instance, the mutation *aristopedia* in *Drosophila* results in the development of a leg structure in place of part of an antenna (34). Such mutants may have a defect either in a controlling enzyme, which fails to recognize a particular controlling sequence, or alternatively they might have an altered controlling sequence which is not recognized by the appropriate controlling enzyme. As a result, cells are channeled into an alternative developmental pathway. It has been shown that a homoeotic mutant can mimic transdetermination in that the developmental abnormality originates in a group of cells rather than in one. But in this case the cells are part of a larger clone, the whole organism, with a particular genetic defect. In a similar way a patch of transdetermined cells could originate from a larger clone derived from a cell with altered DNA.

Conclusions

We are aware that no direct evidence exists for specific modification enzymes in eukaryotes, let alone that such enzymes might exercise control of gene activities. Nevertheless, in view of our almost complete ignorance of the mechanism for the unfolding of the genetic program during development, it seems justifiable to suggest speculative hypotheses that may lead to meaningful experi-

mental approaches, particularly when these hypotheses are based on some of the known features of modification systems in bacteria. It is significant that Sager (35) has argued, from a quite different viewpoint, that restriction and modification mechanisms may exist in higher organisms.

A direct search for specific modification enzymes and modified bases in specific sequences will be difficult, as the number of controlling sequences of any one type in the genome may be only one or a few. Methylases have been identified in sea urchin embryos (36), and there is evidence that the distribution of methyl groups in DNA is not random. It may be significant that the doublet CpG is the most highly methylated (6, 36), but occurs much less frequently than expected from the overall base composition of eukaryotic DNA (37). A search for the transition of cytosine to thymine by methylation and deamination has not so far been successful (38).

Although further study of methylases and the pattern of methylation of certain families of reiterated DNA in different tissues or at different stages of development might well be profitable, we feel that it is unlikely that biochemical studies alone will provide direct evidence for our model. The use of developmental mutants is probably essential, since by comparison with wild-type organisms it may be possible to identify the nature of their biochemical defects. We would predict two general classes of mutant: those with altered controlling sequences, which may be dominant (as in the case of operator constitutive mutations); and those with altered controlling enzymes, which would usually be recessive and obtainable in temperature sensitive form. Analysis of developmental pathways can be assisted by the use of homoeotic mutants, and in this connection we agree with McClintock (39), who has emphasized that, if the ordered processes of development are

deranged, then genes which usually become active at very specific times may instead be activated spasmodically or in random fashion during development. Her studies with maize [for a review, see (40)] have led to the discovery of unstable states and controlling elements. The latter not only control the stability and level of expression of nearby genes, but also transpose from one chromosomal location to another. The possibility of transposition of genetic elements has also been discussed in connection with the problem of immunoglobulin synthesis from genes coding for constant and variable regions (41). Many of the properties of such systems as McClintock's could, we believe, be explained on the basis of repeated sequences of controlling DNA, which could dissociate from and reassociate with several chromosomal sites by means of genetic recombination. What may now be needed is an examination of these genetic elements in a higher organism in which both biochemical and genetic studies can be undertaken.

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