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Selective Silencing of Eukaryotic DNA

A molecular basis is proposed for programmed inactivation or loss of eukaryotic DNA.

Ruth Sager and Robert Kitchin

A diverse set of developmental processes has been described in eukaryotic organisms for which no molecular mechanisms are known. These processes range from the selective (uniparental) inheritance of chloroplast (*1–4*) and mitochondrial (*5–7*) DNA's to chromosome elimination in interspecies somatic cell hybrids (*8–13*), and include the nuclear destruction that follows plasmodial fusion in *Physarum* (*13*), haploidization in barley (*14*), effects of B chromosomes in maize (*15*), heterochromatization and chromosome elimination in insects (*16–34*) and in marsupials (*35, 36*), and X-chromosome inactivation in placental mammals (*37, 38*). These phenomena have in common the selective silencing by inactivation or elimination of specific chromosomes or DNA molecules in the presence of unaffected homologs. We propose that all these phenomena are regulated by the same underlying mechanism: modification and restriction of DNA by enzymes with specificity for particular recognition sites.

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Mechanisms of Modification and Restriction of DNA

DNA modification and restriction (M-R) was discovered in bacterial systems (*39*), where it serves to degrade one DNA, usually of exogenous origin, in the presence of a second DNA, usually that of the host, which remains intact. The molecular basis of this phenomenon, now known for several different bacterial M-R systems (*40*), is the presence of specific nucleotide sequences in DNA, four to eight nucleotides in length, which are recognized by the modification and restriction enzymes. If the modification enzyme acts first, it protects the recognition site by DNA methylation from attack by the restriction enzyme, an endonuclease. If the DNA is unmodified, both strands can be endonucleolytically cleaved by the restriction enzyme at or near the recognition site. Further degradation is then carried out by less specific nucleases. Thus, the M-R system is a powerful means of eliminating particular DNA's (such as invading viral DNA's) in the presence of other DNA's (such as host DNA's) which are preserved. Restriction enzymes have exhibited exquisite precision in their nucleolytic attack on specific recognition sites in DNA, as predicted by Arber and Linn (*40*), and have been used extensively for site-specific cleavage and for sequence analysis of DNA.

The detailed molecular mechanisms of modification and restriction may be somewhat different in eukaryotic and prokaryotic systems. We view M-R enzymes operationally in terms of their effects on DNA, either to protect against or to facilitate subsequent elimination or inactivation.

In parallel with the bacterial systems, we define modification as a secondary change in DNA, requiring the continuing presence of an enzyme to carry out the modification after each round of replication, and restriction as a double-stranded break in DNA. However, we broaden the *consequences* of restriction to include not only immediate degradation, but also the more complex events to be discussed below, including chromosome elimination from the nucleus, intranuclear destruction of particular chromosomes, and heterochromatization. This expanded view of restriction takes account of special options which may be available to eukaryotic chromosomes after suffering a double-stranded break, such as rejoining with one or more added, deleted, or substituted nucleotide pairs. Thus, restriction in this expanded definition may include changes in primary sequence which are subsequently replicated as such.

The nuclear DNA's of higher plants and animals contain 5-methylcytosine, but its function is unknown. Scarano has proposed that the enzymatic methylation of cytosine, followed by an enzymatically reversible G·C (guanine·cytosine) to A·T (adenine·thymine) base transition might provide a molecular signal for differentiation; and he has provided some indirect supporting evidence (*41*). Recently, the potential role in differentiation of enzymatically regulated and reversible methylations and base substitutions has been elaborated by Holliday and Pugh (*42*). Their ingenious models utilize both modification by methylation and enzymatically reversible changes in sequence by base transition. Sequence changes in DNA would seem to be excluded as developmental mechanisms, when the results of nuclear transplantation experiments with amphibia are considered (*43*). However, in the light of the base substitution models proposed by Scarano (*41*) and by Holliday and Pugh (*42*), the role in differentiation of reversible changes in DNA sequence merits reconsideration.

In summary, DNA modification-restriction systems are being recognized as molecular mechanisms with great diversity in their application to many biological processes. This article, however, is limited to those processes involving the selective elimination or heterochromatization of organelle DNA's, nuclear chromosomes, or entire haploid genomes.

Selective Silencing of Eukaryotic DNA's

In eukaryotes, two processes inhibit the functioning of one DNA in the presence of its active homolog: (i) *elimination*, the irreversible loss of DNA molecules or chromosomes (or parts thereof); and (ii) *inactivation* by chromosome condensation, that is, facultative heterochromatization (*19*), a condition inherited clonally in tissues and in cell lines. These processes we refer to collectively as "selective silencing of DNA."

Selectivity is a key feature of the silencing events as they occur in the normal life cycles of insects and mammals. In the coccids and in *Sciara*, for example, it is always the chromosomes of paternal origin that are inactivated or eliminated. And in the marsupials so far described, the sex chromosomes of paternal origin are eliminated in bandicoots or inactivated in kangaroos. Crouse (*30*) has proposed the term "imprinting" to describe the selectivity we have been discussing: the alteration in a chromosome that allows it to be distinguished from its homolog. Chandra and Brown (*44*) have recently reviewed the similarities between the chromosome imprinting systems of the coccids, *Sciara*, and mammals and have especially considered the time and site at which imprinting occurs. In this article we propose a molecular mechanism for imprinting based on the modification and restriction of DNA.

Imprinting has previously been viewed as a single event (*30, 44*). However, on our model, modification and restriction events occur at different times in the life cycle. Selectivity between homologs by M-R enzymes requires that modification occurs when the homologs are spatially separate, in different cells or in different regions within the cell. In bacteria, the host DNA is modified at each replication by an active modification enzyme, while the invading viral DNA is unmodified and open to attack by the restriction enzyme. In the eukaryotic life cycles to be discussed below, the same effect could be achieved if modification enzymes were active in the female germ lines but not in those of the male. The fertilized egg would then contain modified chromosomes from the female parent and unmodified ones from the male. Those chromosomes of the male complement that carried specific recognition sites susceptible to the restriction enzyme would then be available for restriction, which occurs in the fertilized egg (*44*).

The consequences of restriction in eukaryotes are more complex than in bacterial systems: intranuclear destruction, chromosome elimination, or heterochromatization, each programmed for a particular developmental stage. We therefore consider restriction in eukaryotes as a presetting event, a heritable change in DNA which is necessary but not sufficient for subsequent events that may have diverse molecular bases.

Regulation by Selective Destruction of DNA in *Chlamydomonas*

The potency of M-R systems in regulating normal developmental processes in eukaryotes first became apparent during studies of the mechanism of maternal inheritance of chloroplast DNA in *Chlamydomonas*. The strongest experimental evidence yet reported for an M-R system in eukaryotes comes from these studies (*1–4*).

Inheritance of chloroplast DNA in Chlamydomonas. In *Chlamydomonas*, chloroplast genes show maternal inheritance. The chloroplast genome from the mt^+ (female) parent is transmitted to all progeny in sexual crosses, while the homologous genome from the mt^- (male) parent is not transmitted; it disappears soon after zygote formation. The mechanism of this loss has been investigated by a coordinate examination of the transmission of chloroplast genes and of chloroplast DNA's. Three lines of evidence point to regulation by an M-R system.

1) Low doses of ultraviolet irradiation of the female parent (but not of the male) just before mating inhibit the process of maternal inheritance (*1*), resulting in biparental zygotes that transmit both maternal and paternal plastid genomes to their progeny. The effect of ultraviolet irradiation on maternal inheritance demonstrates the presence of a component synthesized in the female parent but regulating the elimination of chloroplast DNA from the male in zygotes. Pretreatment of female gametes with ethidium bromide (10 μg/ml) produced a similar effect (*3*). However, pretreatment of male gametes with inhibitors of chloroplast protein synthesis, such as erythromycin and spiromycin, was more effective in blocking maternal inheritance than was treatment of female gametes. This finding indicated that some component synthesized in the male parent was also involved in the regulation of maternal inheritance (*3*).

2) Further evidence on this point came with the discovery of two mutations, *mat-1* and *mat-2*, that alter the pattern of maternal inheritance (*3*). The *mat-1* mutant gene, in males linked to the mt^- locus, has an effect similar to that of ultraviolet irradiation of the female gametes, namely it inhibits maternal inheritance, and thereby

protects the male chloroplast DNA from elimination in the zygote. The *mat-2* mutant gene, linked to the mt^+ locus, has the opposite effect; it makes maternal inheritance even more pronounced than in the wild type and greatly decreases the effectiveness of ultraviolet irradiation, although the mutant strain carrying *mat-2* is not more resistant to ultraviolet than the wild type, as measured by viability. The *mat* gene functions are discussed below.

3) Direct evidence correlating maternal inheritance of chloroplast genes with the preservation or loss or chloroplast DNA has come from the examination of the fates of chloroplast DNA's from the two parents in zygotes. By prelabeling DNA's differentially with $^{14}NH_4Cl$ or $^{15}NH_4Cl$ in some experiments and with [^{3}H]- or [^{14}C]-adenine in others (*1, 2, 4*), the different fates in the zygotes of chloroplast DNA's from the two parents were monitored. (Nuclear DNA's showed no differential behavior.) Total DNA's were extracted from zygotes at intervals after mating and examined in cesium chloride density gradients. Two characteristic events occurred soon after mating. (i) Chloroplast DNA from the female parent was found to undergo a density shift within 6 hours after zygote formation, from the buoyant density of 1.695 g/cm^3 in CsCl, characteristic of vegetative cells and of gametes, to the lighter density of 1.690 g/cm^3 seen only in zygotes. After replication, this DNA returned to its usual buoyant density. (ii) Chloroplast DNA from the male parent disappeared from CsCl gradients within the first 6 hours after zygote formation.

The density shift is evidence that the chloroplast DNA from the female parent is modified and is thus protected from the action of the restriction enzyme. The homologous DNA from the male parent is degraded by this enzyme. If the density shift results from methylation, as in bacterial modification (*40*), about 5 percent of the DNA would have to be methylated to account for the 0.005 g/cm^3 density shift (*45*).

Further support of this interpretation comes from examining zygote DNA's in experiments in which the selectivity of loss was reversed: after ultraviolet irradiation, and in crosses involving the *mat-1* mutation (*4*). When the female parent was irradiated with ultraviolet before mating, most of the chloroplast DNA recovered from zygotes was of paternal origin; and in crosses with strains carrying the *mat-1* mutation, a similar result was obtained. In both conditions, the survival of chloroplast DNA of paternal origin was paralleled by transmission of chloroplast genes from the male parent.

These lines of evidence suggest the following mechanism: (i) M-R enzymes functioning after zygote formation in the chloroplasts of female and male origin, before the two chloroplasts fuse; and (ii) chloroplast DNA containing recognition sites that are sensitive to these enzymes. The homologous DNA in the female is protected by modification, and unprotected in the male. The effects of ultraviolet irradiation and of the *mat* genes may not be directly upon the M-R enzymes, but upon *mat* gene products, G_1 and G_2, postulated as regulators of M-R activity (*3, 4*). Although the postulated enzymes have not been identified directly, the evidence of coordinate effects of ultraviolet irradiation and of the *mat* genes upon chloroplast DNA and upon the chloroplast genome provides strong support for the M-R interpretation.

Selective Destruction of DNA in Other Organisms

Plastid inheritance in Pelargonium. Selective destruction of organelle DNA's by M-R enzymes may also provide the molecular basis for non-Mendelian inheritance in other organisms (*1*). For example, an apparently complex pattern of non-Mendelian plastid inheritance in *Pelargonium* (*46*) has recently been comprehensively reinvestigated by Tilney-Bassett (*47*). Using crosses between plants containing either normal green or mutant white plastids in their germ layers, Tilney-Bassett has shown that the non-Mendelian pattern of plastid transmission is controlled by a nuclear gene, active in the female parent, and having a direct influence on plastid replication (that is, plastid DNA replication) in zygotes just after fertilization or in early embryos. Some secondary effects on plastid transmission patterns are influenced by the genotype of the male parent.

In broad outline [see (*48*) for a fuller discussion], the *Pelargonium* results resemble those we have described in *Chlamydomonas*. It seems likely that M-R enzymes are also controlling plastid transmission here. In most higher plants, plastid mutations show strict maternal inheritance, rather than the complex biparental patterns of *Pelargonium*. Consequently, it has been widely assumed that maternal inheritance merely reflects the lack of any cytoplasmic transmission by the sperm cells. However, as previously noted (*1*) and again stressed by Tilney-Bassett (*47*), strict maternal inheritance may also be under the control of nuclear genes that determine selective transmission of plastid DNA's.

Transmission of mitochondrial DNA's in yeast crosses. In recent studies of mitochondrial genetics with the use of mutations to drug resistance as markers, three phenomena have been described that complicate genetic analysis: bias, asymmetry, and polarity (*5, 6*). Bias is determined by two separate and distinguishable phenomena: asymmetry and polarity. Asymmetry refers to the difference in frequencies of parental alleles and of recombinant classes recovered as a result of the action of nuclear genes that influence mitochondrial DNA transmission in crosses. Asymmetry affects all mitochondrial markers equally. Polarity on the other hand has only been seen in three of the six loci so far identified genetically in yeast mitochondrial DNA. Polarity is the difference in recovery frequencies of these alleles and of recombinants that result from action at a specific site in mitochondrial DNA called omega (ω). Yeast strains fall into two classes, ω^+ and ω^-, defined by their behavior in crosses. Polarity is not seen in $\omega^+ \times \omega^+$ or in $\omega^- \times \omega^-$ crosses. In $\omega^+ \times \omega^-$ crosses, mitochondrial alleles from the ω^+ parent are found in excess in recombinant progeny. The extent of polarity has provided the basis for mapping these loci in linear order, starting at ω. The closer a locus is to ω, the greater is its polarity.

In a recent review (*6*), Dujon *et al.* have proposed that polarity is the result of a gene conversion process. Starting at ω on the ω^- molecule, the mitochondrial DNA is preferentially degraded, and the strand is restored by copying from the intact ω^+ DNA. Differences in gene frequencies would depend on the length of the DNA segment destroyed before repair processes took over. They do not propose a molecular basis for the enzymatic attack on ω^- DNA. A model assuming deletions or duplications in the ω region leading to excision or repair (or both) has been proposed by Perlman and Birky (*7*) to account for polarity.

In our view, polarity can be explained by the action of modification-restriction enzymes. Thus, the ω region in mitochondrial DNA would represent a recognition site for a pair of M-R enzymes coded by nuclear genes. Formally, the difference between ω^+ and ω^- strains could result either from differences in nuclear gene control of M-R enzyme expression, or from differences in the ω site in mitochondrial DNA. The latter possibility is supported by the findings that the ω^- property is lost in crosses: the diploid clones coming from vegetative multiplication of zygotes are ω^+ as are the four products of meiosis recovered after germination of diploid spores. This result suggests that the ω^- property is a special feature of the mitochondrial DNA of these strains, which renders it vulnerable to the M-R enzymes. A

similar suggestion has recently been made by Callen (*49*).

Nuclear destruction in Physarum. Carlile (*13*) has described the nuclear destruction that follows fusion between plasmodia of different strains of *Physarum polycephalum*. For example, fusion between strains 15 and 29 leads to a lethal interaction in which nuclei of strain 29 are eliminated, as shown by cytological observation and loss of genetic markers, while nuclei of strain 15 survive and multiply. Carlile notes that other inhibitory mechanisms also exist, but that "post-fusion lethal interactions are best interpreted as the *final barrier* to fusion of genetically unlike somatic cells."

Chromosome destruction in higher plants. The recent discovery of a method to produce haploid barley seedlings provides a new example of selective chromosome destruction. Interspecies reciprocal crosses between *Hordeum vulgare* (commercial barley) and *H. bulbosum*, each with a diploid chromosome complement of 14, lead to selective elimination of the *H. bulbosum* set, and the recovery of haploid plants containing only the seven chromosomes from *H. vulgare* (*14*).

The results are consistent with the operation of an M-R system, coded by the *H. vulgare* genome and active against the *H. bulbosum* chromosomes. Such a proposal has been made by Davies (*50*), who also suggested that ultraviolet irradiation and prior treatment with inhibitors could be used to look for evidence of an M-R system in barley, following the approach used in studies of chloroplast DNA elimination in *Chlamydomonas*.

Rhoades and Dempsey (*15*) have described a naturally occurring system of chromosome elimination in maize, regulated by the B chromosomes, which are a set of nonessential heterochromatic chromosomes present in some maize strains. The B chromosomes were shown to interfere with normal replication, leading to chromosome breakage and elimination of those A (essential) chromosomes that contain a heterochromatic knob. Elimination occurs only at a single cell division in the life cycle, during pollen formation, and may therefore be susceptible to experimental manipulation. Here, restriction is seen as "stickiness" of heterochromatic B's and knobs, leading to nondisjunction, breakage, and elimination.

Intranuclear chromosome destruction in an insect. In male coccid insects, selective silencing of the entire paternally derived haploid set of chromosomes is a central feature of the life cycle. The silencing process is achieved by facultative heterochromatization or by nondisjunction leading to elimination in most coccids (as is discussed below and Fig. 1). However, during spermatogenesis in certain genera, one or more heterochromatic chromosomes suddenly disappear from each primary spermatocyte. This loss is the result of intranuclear destruction, as described, for example, in the olive scale insect *Parlatoria oleae* (*25*). The destruction of particular chromosomes within the nucleus, in the presence of their homologs which remain intact, is a prime example of a phenomenon for which an M-R process would seem uniquely appropriate.

Regulation by Chromosome Elimination or Heterochromatization

Of the three known modes of selectively silencing long multigenic stretches of DNA, we have discussed the one where there is outright destruction. We turn now to elimination and facultative heterochromatization. Logically, these two modes might be discussed separately, since they appear to be so different. We have found it preferable to consider them together, because both modes occur as alternatives in the life cycles of closely related Hemiptera, notably the coccids (*17–20, 22, 25, 26*). In addition, facultative heterochromatization of the X chromosome is common in both marsupials (*35*) and in placental mammals (*37*), but has been replaced by chromosome elimination in one group of marsupials, the ratlike bandicoots (*36*). In all of these organisms, the function of chromosome elimination and of heterochromatization appears to be the same: either to function in sex determination or to maintain the proper gene balance between the sex chromosomes and autosomes in chromosomal sex determination (dosage compensation).

The Coccids

Three clearly different chromosome systems in the coccids—the lecanoid, *Comstockiella*, and diaspidid—which are characterized by heterochromatization or elimination of chromosomes in the males, have been described (Fig. 1). In both the lecanoid and *Comstockiella* systems, male and female zygotes begin development as true diploids; but during early embryonic development in the males the paternally derived set of chromosomes becomes heterochromatic and functionally inactive and remains so in most tissues throughout development (*18, 19, 21, 23–25*).

In the evolutionarily more advanced diaspidid system (for example, in some armored-scale insects) the males are true haploids. The paternal set of chromosomes fails to divide and is lost from each cell very early in development in those individ-

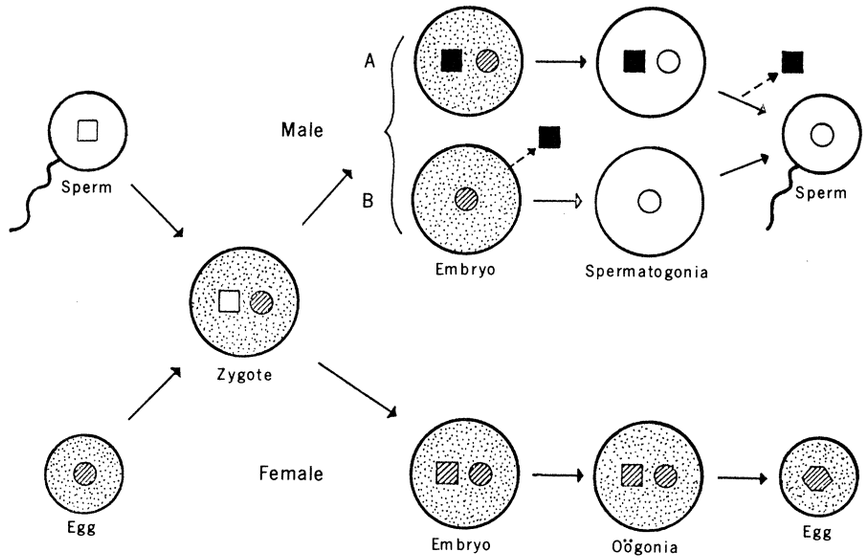

Fig. 1. Modification-restriction in the coccids. The fertilized egg contains a modified set of chromosomes from the mother and an unmodified set from the father. If the paternal set is modified, the embryos become females. The modification enzyme is active (stippled cytoplasm) throughout development in all tissues in females. If the paternal set is restricted, the embryos become males. In the lecanoid and *Comstockiella* systems (A), the restricted set becomes heterochromatic and is retained until spermatogenesis and is then eliminated. In the diaspidid system (B), the restricted set is eliminated during embryonic development. Because the modification enzyme is not active in the male germ line, the chromosome set transmitted by the male is unmodified. Sex determination is dependent on whether the unmodified chromosomes from the sperm are modified or restricted after fertilization. A, lecanoid and *Comstockiella* systems; B, diaspidid system; □, genome of paternal origin; ○, genome of maternal origin; ◌, genome of either maternal or paternal origin; ▨, modified DNA; and ■, restricted DNA becomes heterochromatic or is eliminated.

uals destined to become males (*20*). The comparison between these systems is particularly instructive because the same biological result, effective haploidization of the male, is accomplished by elimination of the paternal chromosome set in the diaspidid system, and by its heterochromatization in the other two systems. The close parallel in life cycles, as indicated in Fig. 1, suggests that the same imprinting mechanism occurs in the three coccid systems resulting in either heterochromatization or chromosome elimination.

In species with the lecanoid system, the paternally derived heterochromatic chromosomes are eliminated during a modified spermatogenic sequence and slowly disintegrate in the cytoplasm. In the *Comstockiella* system one or more paternally derived heterochromatic chromosomes are destroyed within the nucleus of each primary spermatocyte shortly before meiosis (for example, *Parlatoria oleae*, as described above); the remaining heterochromatic chromosomes are eliminated during spermatogenesis. In the diaspidid system, in which the males are haploid, spermatogenesis is a simple mitotic division, producing two functional sperm each carrying a complete set of maternally derived chromosomes. Thus, after spermatogenesis in all coccid males the functional sperm transmit only the maternally derived euchromatic set of chromosomes.

The proposed operation of an M-R system for the coccid life cycle is shown in Fig. 1. The fertilized egg contains a modified set of chromosomes from the female parent and an unmodified set from the male. The sex of the embryo is determined by the M-R enzymes at the time of fertilization. If the chromosomes of male origin encounter the modification enzyme before the restriction enzyme in the egg cytoplasm, they will be modified and the embryo will develop as a female; if they are restricted, the embryo will develop as a male. At the fifth to eighth cleavage division, the restricted set will become heterochromatic (in the lecanoid and *Comstockiella* systems) or be eliminated (in the diaspidid system). In females the M-R enzymes are active in the germ line, and consequently the chromosomes of the haploid egg nucleus are always modified.

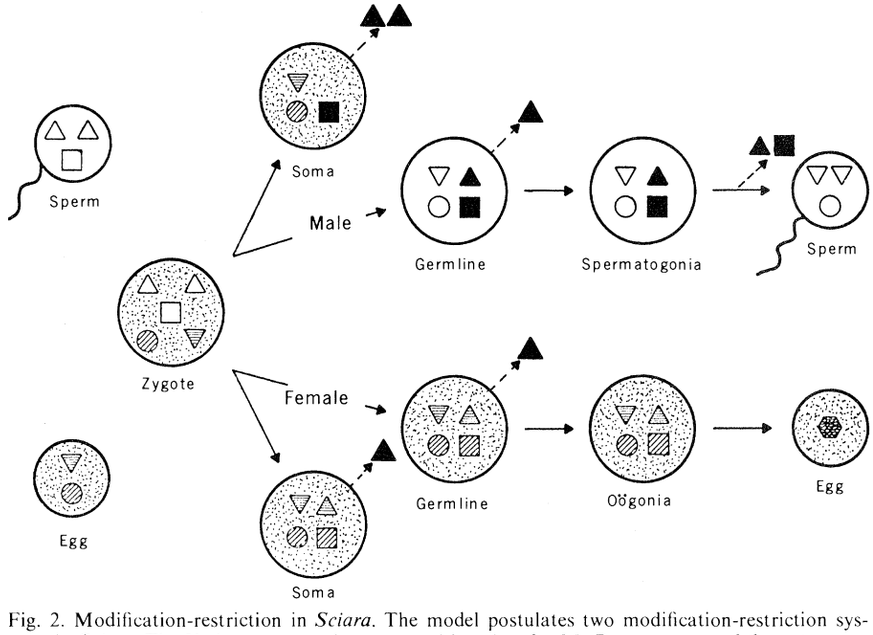

Fig. 2. Modification-restriction in *Sciara*. The model postulates two modification-restriction systems in *Sciara*. The X chromosomes have recognition sites for M_1-R_1 enzymes, and the autosomes have recognition sites for the M_2-R_2 enzymes. Modification of X is indicated by horizontal cross-hatching and autosomal modification by vertical cross-hatching. Both M-R systems are active in male and female somatic cells and are also active in the female germ line but not in the male germ line. The fertilized egg receives a modified set of autosomes and one X chromosome from the mother and an unmodified set of autosomes and two X chromosomes from the father. If the paternal autosomes and one paternal X are modified by the M_1 and M_2 enzymes the embryos will develop as females. If the paternal chromosomes are all restricted, the embryos will develop as males. The restricted X chromosome (or chromosomes), one in females and two in males, are eliminated from the somatic cells in early development. In the germ line only one restricted paternal X chromosome is eliminated in both sexes. Meiosis is normal in females. Spermatogenesis, however, is unusual and only the maternally derived autosomes and two maternal X chromosomes are transmitted by each sperm. Because the M-R systems are not active in the male germ line, the chromosomes transmitted by the male are unmodified and can subsequently be modified or restricted in the fertilized egg. ▽, Maternal X chromosome; △, paternal X chromosome; ○, maternal autosome; [ ], paternal autosome; ⬡, maternal and paternal chromosomes; ≡, X-chromosome modification; ⫽, autosome modification; open symbols, unmodified; closed symbols, restricted; →, eliminated.

In parthenogenetic coccids (*51*), an unfertilized haploid egg nucleus may divide once mitotically to form two genetically identical daughter nuclei. These nuclei later fuse to form a homozygous diploid zygote. In about 5 percent of these embryos one of the two haploid sets becomes heterochromatic, and the embryos develop as males despite the fact that both chromosome sets are of maternal origin. Chandra and Brown (*44*) have concluded that in the parthenogenetic coccids one of the two haploid division products of the egg nucleus was imprinted in the egg cytoplasm during the brief period in which the two nuclei were separated from each other.

In terms of the M-R model of imprinting, restriction enzymes may be present in some eggs and absent in others; or there may be spatial or temporal variation in the amount of restriction enzymes present in the egg. The mealybug egg is relatively large (0.40 by 0.25 mm) and the imprinting region within the egg seems to be localized (*44*). Also, the changes in sex ratio described in several coccids and correlated with maternal age (*52*) are compatible with temporal changes in the amount of restriction enzymes present in the egg.

Reversal of heterochromatization in coccids. Heterochromatization is induced and maintained by genes active in the euchromatic set and, at least in the coccids, is a reversible state that is under developmental control. Reversal of heterochromatization occurs regularly during development in male mealybugs and some other coccids (*23*). The tissues affected vary from species to species. Reversal has been induced experimentally by treating living mealybug testis explants with a synthetic polyanion. This treatment removes specific chromosomal histones from isolated calf thymus nuclei (*53*) and results in transcriptional activity of heterochromatin (*54*). Reversal of heterochromatization in the coccids could result from reversible changes in the nucleotide sequence of the M-R recognition sites, as proposed by Scarano (*41*) and Holliday and Pugh (*42*), or by developmentally programmed changes in the activity of genes that control heterochromatization and that are located in the euchromatic set.

Sciara

The cytogenetic system in *Sciara*, which was first analyzed by Metz (*27, 29*) and later by Crouse and her students (*30–32*), is quite complex. Both male and female zygotes begin development with two sets of autosomes and three X chromosomes, two of which are inherited from the father. Chromosome elimination first occurs dur-

ing early cleavage and marks the separation of the soma from the germ line, as diagrammed in Fig. 2. For example, in *Sciara coprophila* the X chromosomes of paternal origin, one in females and two in males, are eliminated from all somatic cells during the fifth to eighth cleavages (*28*). Later in development one paternal X chromosome is eliminated from the germ line of both sexes.

Oogenesis is normal in *Sciara*, and the egg receives a haploid set of autosomes and one X chromosome (*32*). In spermatogenesis, however, the single functional sperm formed contains two maternal X chromosomes and a haploid set of maternally derived autosomes (*27, 32*). The paternally derived X and autosomes are discarded. Thus, in *Sciara*, as in the coccids, the parental origin of the chromosomes determines their future fate in both the somatic cells and in the germ line. The male transmits through the sperm only the chromosomes that he inherits from his mother.

Genetic evidence reviewed recently by Chandra and Brown (*44*) indicates that in *Sciara* the sex of the zygote is determined by its chromosomal constitution. The genetic constitution of the mother determines the sequence of chromosome eliminations that will occur in the zygote. Two types of females have been described in *Sciara*: XX′ and XX. Although a single male can inseminate females of both types, XX′ females usually produce only daughters and XX females usually produce only sons. Because *Sciara* males transmit the same chromosomes to both their sons and daughters, the paternal chromosomes must enter the egg unaltered and chromosome imprinting in *Sciara*, as in the coccids, must occur within the egg.

We propose that DNA modification-restriction is the molecular mechanism underlying imprinting in *Sciara*. However, because imprinting is far more complex in *Sciara* than in the coccids we can discuss only the basic features of our M-R model here.

Two M-R systems are required in *Sciara* to distinguish between the X chromosomes and autosomes, as well as between the maternal and paternal chromosome complements. The X chromosomes carry recognition sites for the M_1-R_1 enzymes, and the M_2-R_2 enzymes specifically recognize the autosomes. We postulate that both of these systems are active in male and female somatic cells prior to chromosome elimination, and in the female germ line but not in the male germ line. Multiple restriction systems have been identified in bacteria (*40*), providing a precedent for this assumption.

The egg nucleus contributes one X chromosome modified by M_1 and a haploid set of autosomes modified by M_2; the sperm contributes two X chromosomes and one autosomal set unmodified and susceptible to restriction by R_1 and R_2, respectively (Fig. 2). If the chromosomes derived from sperm are modified by the M_1 and M_2 enzymes, the fertilized egg develops as a female; if the sperm chromosomes are restricted, the embryo develops as a male. *Sciara* species with XX′ female producers and XX male producers may have an additional M-R system active in the germ line of XX′ females, which modifies the X chromosome in the haploid egg nucleus prior to fertilization and allows the maternal and paternal modified X chromosomes to be distinguished in female zygotes. The behavior of the X chromosome in *Sciara* is governed by a block of heterochromatin very close to its centromere. X-autosome translocations containing the heterochromatic controlling element behave like X chromosomes while those without it behave like autosomes (*30*). We suggest that this heterochromatic region contains recognition sites for the M-R enzymes.

Mammals

A type of X-chromosome inactivation occurs in the marsupials. However, at least in the kangaroos, the choice is not random; it is the paternally derived X that is inactivated (*35*). Thus, in marsupials, as in *Sciara* and the coccids, an imprinting mechanism must exist to distinguish the chromosomes of maternal and paternal origin.

Remarkably, sex chromosome elimination replaces inactivation in some marsupial bandicoots (Fig. 3). In these ratlike species, the sex chromosome constitution is always XX or XY in the germ line, but some somatic tissues have only a single X chromosome as a result of elimination early in development of one X in females and of the Y in males (*36*). The eliminated X is probably of paternal origin. The formal similarity between the elimination schemes in Figs. 1 and 3 is remarkable. The only known difference between the coccid and marsupial models is the limitation of M-R enzyme recognition sites to the *sex chromosomes* in marsupials.

The occurrence of X-chromosome in-

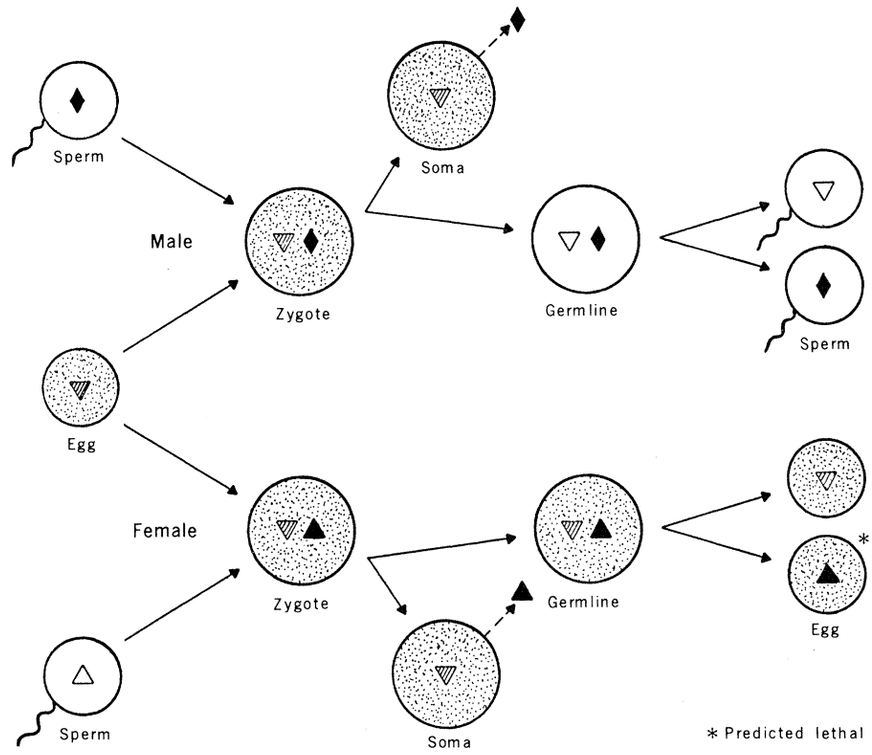

Fig. 3. Modification-restriction in marsupials. The paternally derived sex chromosome is heterochromatic and genetically inactive in most marsupials and is eliminated from some somatic tissues in others, such as bandicoots. The Y chromosome is male determining. The model postulates that receptor sites for the modification and restriction enzymes are present only on the sex chromosomes. The M-R enzymes are active (stippled cytoplasm) in the somatic cells of both sexes and in the germ line in females only. Because the M-R system is not active in the male germ line, the maternally derived X is transmitted unmodified in the sperm. If the changes induced by the restriction enzyme are irreversible, the paternally derived Y transmitted by the male and the paternally derived X transmitted by the female will be restricted. Females with two restricted and genetically inactive X's would presumably die. ▽, Maternal X chromosome; △, paternal X chromosome; ◆, Y chromosome; ⫽, modified; closed symbols, restricted; open symbols, unmodified.

activation in female placental mammals was discovered by Lyon on the basis of genetic evidence (*37*) and subsequently correlated with the Barr body phenomenon, that is, the heterochromatic state of one X chromosome in female somatic cells. It is now well established that the choice of which X chromosome becomes inactivated by heterochromatization occurs at random in each cell, early in embryonic development, and is heritable in the clonal descendants of these cells. Thus, female mammalian tissues are fine-grained mosaics for X-linked heterozygous genes. Presumably the function of X-chromosome inactivation is dosage compensation, to maintain the same ratio of X chromosome to autosome in both males and females.

The special feature of X-chromosome inactivation in placental mammals is the random choice of which X becomes heterochromatic. Brown and Chandra (*38*) have proposed that the randomness is an evolutionary advance on the mechanism of paternal X inactivation seen in marsupials. Their model for mammals involves a two-part control system: a sensitive site subject to imprinting, which activates a receptor site, which, in turn, regulates heterochromatization of the X chromosome. In marsupials, the two sites are adjacent on the X chromosome, and inactivation is determined by prior imprinting. In placental mammals, Brown and Chandra (*38*) propose that the sensitive site has been translocated to an autosome.

M-R enzymes could provide the molecular basis for imprinting. The sensitive site would be subject to prior modification and restriction (Fig. 3) resulting in availability of one and not the other homologous locus for activation at a programmed time in development. Activation of the sensitive site would then produce a gene product that would activate the receptor site on one X chromosome to protect it from heterochromatization.

Chromosome Elimination in Cell Hybrids

Preferential chromosome elimination in interspecies somatic cell hybrids was first reported by Weiss and Ephrussi (*8*) in hybrids between rat and mouse cells. The frequent loss of human chromosomes in hybrids with mouse, rat, and hamster cells has proved a powerful means for locating genes on particular human chromosomes (*9*). More than 100 genes of the human genome have now been assigned to particular chromosomes by following the concordant loss of chromosomes and phenotype in certain interspecies cell hybrids. Recently, however, a major reversal of polarity of chromosome loss, previously reported as a rare event (*10*), has been discovered in hybrids between embryonic rodent cells and the human cell line WI-18-VA2 (an SV40-transformed subline of WI-18 that is deficient in hypoxanthine phosphoribosyltransferase) (*11*).

The only difference so far known between these studies and those of other investigators that could account for the reversal of polarity of chromosome elimination is the source of parental cells. Conventionally, established rodent cell lines are used, whereas in these studies the rodent cells were freshly isolated from embryonic nervous tissue.

Reversal of polarity clearly rules out one of the proposed mechanisms of preferential chromosome elimination, namely spindle specificity. Other proposed mechanisms such as differences in growth rates, differences in stages of the cell cycle, and random nondisjunction, have been criticized rather persuasively by Handmaker (*12*). One proposal, preferential nondisjunction, remains open to further consideration.

A modification-restriction system could provide the initial event that determines preferential nondisjunction by affecting the centromere or by altering some other feature of the chromosome essential for normal disjunction. No detailed description of the initial chromosome elimination process that sheds light on the mechanism has yet been reported.

On the M-R hypothesis, it is assumed that species differ in particular M-R enzymes and recognition sites that can determine chromosome elimination. For example, rodent M-R enzymes may have more specificity for sites on human chromosomes than do human M-R enzymes for rodent chromosomes. These enzymes may be weak or unexpressed in embryonic tissues, thus accounting for the survival of human chromosomes in the experiments of Horak *et al.* and Minna and Coon (*11*). If this hypothesis has validity, it should be possible to test it at the cellular level by suitable treatments of the parental cells before fusion in a manner analogous to the manipulation of chloroplast DNA elimination in *Chlamydomonas*, such as by ultraviolet irradiation, by inhibitors of protein synthesis or transcription, or by the selection of mutations that influence the process, and ultimately by direct isolation of the M-R enzymes.

Concluding Remarks

The modification and restriction of DNA is proposed as the underlying molecular mechanism for a diverse set of genetic phenomena that involve the loss or inactivation of DNA molecules, whole chromosomes, or entire haploid sets. In organelle DNA's the enzymatic mechanisms of DNA elimination may be very similar to those in bacteria, since the DNA's are relatively protein-free. In the nucleoprotein complexes of nuclear chromosomes, the specific enzymatic mechanisms of degradation and elimination may differ markedly from those operating in bacteria.

In each organism discussed, modification is postulated to protect the modified DNA from nuclease action by the corresponding restriction enzyme, and to depend upon the continuing activity of the modification enzyme. The action of the restriction enzyme is postulated as a presetting mechanism, generating a small inherited change in DNA that potentiates later degradation or heterochromatization.

The value of DNA modification-restriction as a unifying hypothesis is as follows: (i) It provides a common molecular mechanism for an apparently great diversity of phenomena. (ii) It provides a basis for the design of experiments to test the hypothesis. (iii) In testing the hypothesis, experiments may provide clues leading to the successful manipulation and control of chromosome elimination in somatic cell hybrids. Finally, the existence of M-R systems regulating chromosome behavior increases the likelihood that this mechanism also plays a role in other aspects of eukaryotic development, including the regulation of normal patterns of growth.

Note added in proof: Restriction enzyme activity has been detected in extracts of *Chlamydomonas*, vegetative cells, gametes, and zygotes assayed with adeno-2 viral DNA (*55*).

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The Coming of Age of the Cell

The inventory of cells by fractionation, biochemistry, and electron microscopy has affected our status and thinking.

Albert Claude

Fifty years of cell research can hardly be summarized in the 20 to 30 minutes of a lecture; to expose only part of it might be unrepresentative, unfair, and altogether unnecessary, since by now you have already been informed of the essential facts and discoveries that have accumulated in the course of these years.

What I would like to do instead is to discuss with you the impact of these discoveries on our daily life and their significance for the present and the future. At the same time I will try to recall, firsthand, what has been my own experience in this century's endeavor to uncover what were, not so long ago, the mysteries of life itself.

Until 1930 or about then, biologists were in the same situation as astronomers and astrophysicists, who were permitted to see the objects of their interest, but not to touch them; the cell was as distant from us as the stars and galaxies were from them. More dramatic and frustrating was that we knew that the instrument at our disposal, the microscope, so efficient in the 19th century, had ceased to be of any use, having reached, irremediably, the theoretical limits of its resolving power.

I remember vividly my student days, spending hours at the light microscope, turning endlessly the micrometric screw, and gazing at the blurred boundary which concealed the mysterious ground substance where the secret mechanisms of cell life might be found. At last I remembered an old saying, inherited from the Greeks—that the same causes always produce the same effects—and I realized that I should stop that futile game and try something else. In the meantime, I had fallen in love with the shape and the color of the eosinophilic granules of leukocytes and attempted to isolate them. I failed—and consoled myself later on in thinking that this attempt was technically premature, especially for a premedical student, and that the eosinophilic granules were not pink, anyway. The isolation was only postponed. That Friday, 13 September 1929, when I sailed from Antwerp on the fast liner *Arabic* for an 11-day voyage to the United States, I knew exactly what I was going to do. I had mailed beforehand to Simon Flexner, director of the Rockefeller Institute, my own research program, handwritten, in poor English, and it had been accepted. My proposition had been to isolate and determine by chemical and biochemical means the constitution of the Rous chicken tumor I agent, at that time still controversial in its nature and not yet recognized as a bona fide virus. This task occupied me for about 5 years. Two short years later the microsomes, basophilic components of the cell ground substance, had settled in one of my test tubes, still a structureless jelly, but now captive in our hands.

In the following 10 years, the general method of cell fractionation by differential centrifugation was tested and improved, and the basic principles were codified in two papers in 1946. This attempt to isolate cell constituents might have been a failure if they had been destroyed by the relative brutality of the technique employed. But this did not happen. The subcellular fragments, obtained by rubbing cells in a mor-

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The author is professor at the Université Catholique de Louvain, Belgium, director of the Laboratoire de Biologie Cellulaire et Cancérologie at the Université Catholique de Louvain, and adjunct professor at Rockefeller University, New York 10021. This article is the lecture he delivered in Stockholm, Sweden, on 12 December 1974 when he received the Nobel Prize for Physiology or Medicine, a prize he shared with George Palade and Christian de Duve. The article is published here with the permission of the Nobel Foundation and will also be included in the complete volume of *Les Prix Nobel en 1974* as well as in the series Nobel Lectures (in English) published by the Elsevier Publishing Company, Amsterdam and New York. The lecture by de Duve appeared in the 18 July issue, and the lecture by Palade appeared in the 1 August issue.