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FIRST STEPS IN EUKARYOGENESIS:
Physical phenomena in the origin and evolution of chromosome structure

Julian Chela-Flores (+)
International Centre for Theoretical Physics,
Miramare P.O.Box 586; 34100 Trieste, Italy
and
Dublin Institute for Advanced Studies,
10, Burlington Road, Dublin 4, Ireland.

Abstract.

Our present understanding of the origin and evolution of chromosomes differs considerably from current understanding of the origin and evolution of the cell itself. Chromosome origins have been less prominent in research, as the emphasis has not shifted so far appreciably from the phenomenon of primeval nucleic acid encapsulation to that of the origin of gene organization, expression, and regulation. In this work we discuss some reasons why preliminary steps in this direction are being taken. We have been led to examine properties that have contributed to raise the ancestral prokaryotic programmes to a level where we can appreciate in eukaryotes a clear departure from earlier themes in the evolution of the cell from the last common ancestor. We shift our point of view from evolution of cell morphology to the point of view of the genes. In particular, we focus attention on possible physical bases for the way transmission of information has evolved in eukaryotes, namely, the inactivation of whole chromosomes. The special case of the inactivation of the X chromosome in mammals is discussed, paying particular attention to the physical process of the spread of X inactivation in monotremes (platypus and echidna). When experimental data is unavailable some theoretical analysis is possible based on the idea that in certain cases collective phenomena in genetics, rather than chemical detail, are better correlates of complex chemical processes.

(+) Also at the Instituto Internacional de Estudios Avanzados (Universidad Simon Bolivar). Apartado 17606 Parque Central. Caracas 1015A, Venezuela.

1. Eukaryogenesis. A gene-centred approach.

1.1.EVOLUTION OF CELL MORPHOLOGY AND CHROMOSOME STRUCTURE

The paleontological record suggests that the origin of the nucleated or eukaryotic cell (eukaryogenesis) occurred earlier than 1,500 million years before the present (Mybp). Some algae may even date from 2,100 Mybp [1]. This is still rather late, compared to the earliest available prokaryotic fossils to which a date of 3,500 Mybp has been assigned [2]. Regarding the appearance of more ancient eukaryotes than the above-mentioned Paleoproterozoic algae, we should remember that it may be difficult to explain the existence of such ancient eukaryotes in the period of banded-iron formation, which ended 1,800 Mybp [3]. One particular difficulty is presented by the known abundance, prior to 2,300 Mybp, of the easily oxidized mineral form of uranium (IV) oxide (urininite, *pitchblende*.) Only when the oxygen sink (ferrous iron) was exhausted, was it possible for concentrations of free oxygen to begin increasing in the atmosphere. The onset of atmospheric oxygen is demonstrated by the presence in the geologic record of red shale coloured by ferric oxide: such 'red beds' are estimated to be some 2,000 milion years old (Orosirian Period of the Paleoproterozoic).

We may not exclude from the geochemical data earlier dates, in the lower Archean, for the first prokaryotic microflora [4], although some considerations from the point of view of geochronology should be kept in mind [5].

Once the eukaryotes enter the fossil record, its organization into multicellular organisms followed in a relatively short period (in a geological time scale.) Metazoans are hypothesized to have arisen as part of a major eukaryotic radiation in the Riphean Period, approximately 800-1,000 Mybp [6]. There is some evidence in the Neoproterozoic, in Vendian time, for the existence of early diploblastic grades (Ediacaran faunas). These organisms were early metazoans with two germ layers, such as the modern coelenterates. Later on these grades were overtaken in numbers by triploblastic phyla (Cambrian faunas, which were mainly metazoans with three germ layers) constituting at present the greater majority of multicellular animals. We may obtain further insights from paleontology: acceleration in the evolutionary tempo is observed after the onset of eukaryogenesis, as it is clearly demonstrated by the microfossils of algae from the Neoproterozoic [6] and by the macrofossils of the early Phanerozoic (Cambrian Period) [7]. It is possible that such evolutionary changes may have had counterparts in corresponding changes in the eukaryotic genome. A possible candidate for such a counterpart is chromosome plasticity (cf., Sec. 3.) This conjecture will be explored in the present work (in Secs. 4 and 5.)

Together with evolution in cellular morphology, there is corresponding evolution in structure, organization, and genetic regulation of the DNA in the nucleoid of

prokaryotes. The simplest chromosomes, and possibly the earliest [8], are those of viroids and plasmids. Complexity, understood as increments in gene-expressing nucleic acid, increases from the RNA viroid level to the DNA prokaryotic chromosome (PC), found in the more evolved archaebacteria, eubacteria, chloroplasts, and mitochondria. However, maximum complexity is only reached with the first appearance of the eukaryotic chromosome (EC). The consideration of the evolution from the PC to the EC is forced upon us when we look closer at properties of the contemporary genome of the living cell.

1.2. SOME DISTINCTIVE FEATURES OF CHROMOSOMES

The PC is a double-stranded DNA structure usually lacking:
1.2.1. Abundant packaging proteins (histones).
1.2.2. An enveloping membrane.
1.2.3. Different specialized regions, such as the nuclear organelle, associated with the site of ribosomal RNA-coding genes (nucleoli).
1.2.4. Ends formed by highly repeated sequences (telomeres).
1.2.5. Shut-down inhibition of gene expression (gene silencing). This may involve whole chromosomes, leaving some exceptional loci with the ability to transcribe pre-messenger RNAs (pre-mRNAs.) PCs consist of a beaded structure, not unlike that of the EC [9]. Even histone-like proteins are known in some prokaryotes: *Escherichia coli* [10, 11], Cyanobacteria [12], the short rod-shaped human pathogen *Pseudomonas aeruginosa* [13], and the obligate sexually-transmitted intracellular human parasite *Chlamydia trachomatics* [14].

We cannot argue in favour of a clear-cut difference between PCs and ECs from the point of view of genome size. Indeed, although PCs are normally smaller than ECs, some eukaryotes have very small chromosomes. One example is provided by the Rhodophyte *Cyanidioschyzon*. This seaweed has a genome of only 8 million (M) base pairs (bp) [15]. This tiny genome is only twice as long as the corresponding one in *E. coli* (3.5 Mbp.)

On the other hand, the problem of eukaryogenesis is rendered still more difficult to define, as the EC has some characteristics which are not common to all eukaryotes. Some exceptions are particularly remarkable in lower eukaryotes, such as algal protists: in these cases we are faced with chromosomes lacking histones. For instance, in the dinoflagellates *Blastodinium* Chatton and *Amphidinium elegans* [16] there are distinct chromosomes which are not associated with histones. *Prorocentrum micans* is a neurotoxin-producing marine dinoflagellate that occasionally may cause local outbreaks of extremely devastating red water; its chromosomes have no 'beaded' structure, which normally are due to sets of histones being complexed with DNA (such structures are called 'nucleosomes') [17]. Furthermore, the

absence of histones is conspicuous in other eukaryotes, such as in three genera of fungi *Microsporum, Neurospora and Phycomyces* [18].

For the above reasons only an exceptional group of eukaryotic chromosomes may be considered primitive [19]. Consequently, the origin and evolution of chromosomes becomes a relevant investigation in origin-of-life studies. The most likely cause for the evolution of complex chromosome structure seems to be regulation of gene expression, a process which has reached its maximum expression in eukaryotes (cf., Sec. 4)[20].

For a considerable time now, it has been evident that the integration of proteins complexed with DNA ('chromatin') has played a fundamental role in the regulation of gene expression [21]. Some chromatin replicates its DNA late in the S phase of the cell cycle (cf., Sec. 2.2); it is also dark-staining, due to the high degree of its DNA packaging. In order to differentiate such a special state of chromatin from its less dense counterpart ('euchromatin'), we refer to chromatin in the highly packed case as 'heterochromatin'.

However, it is convenient to introduce the concept of a dense form of chromatin which could be due to its specific DNA sequence. One such instance of chromatin contains highly repetitive DNA, which is associated with heterochromatization. This point will be considered below, in Sec. 3.2, in our discussion of 'satellite DNA'. A closely related state of chromatin which is the result of regulation rather than structure, is sometimes found in a higher state of DNA packaging. Such chromatin is referred to as 'facultative heterochromatin'. We reserve the term 'constitutive heterochromatin' to chromatin that finds itself in a dense state of packaging due to its permanent structure. In the fruitfly *Drosophila melanogaster*, a specific non-histone protein (HP-1) is known to influence directly chromatin structure [22]; such protein may suppress the inactivation of gene expression in constitutive heterochromatin, demonstrating that such a protein could participate in a typically eukaryotic shut-down mechansim of chromosomes.

2. Evolution of DNA synthesis and gene regulation

2.1. ORIGINS OF EUKARYOTIC DNA REPLICATION AND TRANSCRIPTION

Two of the central pathways of macromolecular synthesis are relevant to our discussion, namely, DNA synthesis and transcription of pre-mRNA. These processes are well established in prokaryotes and were further elaborated by eukaryotes, generally increasing their complexity. In some cases some radical departures were initiated which, from the point of view of the genome, may be considered as true hallmarks of eukaryogenesis.

Some thirty years ago the 'replicon' model was introduced in an effort to understand bacterial DNA replication in

terms of units of replication, the so-called 'replicons' [23]. The main themes of this model are:
2.1.1. A structural gene controls the synthesis of a specific protein, or 'initiator', which is involved in the initiation of DNA replication and,
2.1.2. A single origin of replication (i.e., the single target sequence recognised by the initiator) allows the starting of replication. In *E. coli*, for instance, the corresponding sequence 'ori-C' has 245 bp [24].

More complex eukaryotic DNA replication follows the guidelines identified in prokaryotes, but differs in some essential aspects:
2.1.3. A considerably richer repertoire of enzymes is needed for the generally larger eukaryotic genome [25].
2.1.4. Multiple origins of replication are spaced at an average of 50-100 thousand base pairs (kbp) [26, 27].
2.1.5. Origins are activated at different times in the S phase of the cell cycle, but adjacent origins are activated at about the same time [28, 29].

Once again, in eukaryotes we find that the repertoire of enzymes required for RNA synthesis exceeds by far the simpler set needed in bacterial transcription. In the process of the eukaryotic elaboration of earlier themes, nevertheless, the coupling between transcription and DNA replication is strictly preserved [30]. The main point we wish to emphasize here is that sets of adjacent genes transcribed collectively into a single pre-mRNA ('operons') may contain genes required for the initiation of transcription, as well as genes that may play a role in DNA replication. We may also find the opposite situation, transcriptional factors may be components of eukaryotic origins of replication [24].

2.2. HETEROCHROMATIN: A HALLMARK OF EUKARYOGENESIS

In the process of transcription more complexity is introduced by evolutionary mechanisms, as the RNA polymerase requires an array of activators, coactivators, and basal factors which, for instance, go well beyond the relatively simple set of sigma factors of *E. coli*. This set of enzymes collects on the sequence recognised by the RNA polymerase as the site to begin transcription (the 'core promoter') [31], and may be considered analogous to the *E. coli* sigma factors. Unlike the simple bacterial strategy for synthesizing RNA transcripts, eukaryotes have neither simple adjacent controlling elements ('promoters'), nor DNA sequences that inhibit transcription ('operators'.) Instead, RNA polymerases cannot work, in the case of eukaryotes, entirely with adjacent elements, but need to orchestrate their activity with distant segments (measured in kbp) called 'enhancers' and 'silencers' which, in turn, require their own set of transcription factors [32].

We return to the replicon model in our search for typical mechanisms brought about in evolution by the requirements

for DNA replication. In the numerous replication origins we may find a hint of such a typical eukaryotic mechanism. The temporal order for the initiation of origins is not controlled by a property of the origin itself; but a likely candidate for controlling this aspect of DNA replication in eukaryotes is control at the level of chromatin structure. In this context, as mentioned in Sec. 1.2 for over three decades it has been well known that genes on heterochromatin go through DNA replication late in the cell cycle [33]. We return to this topic in Sec. 5.1, in order to rationalize the phenomenon of late DNA replication of heterochromatin.

3. Regulation of facultative heterochromatin

3.1. CHROMOSOME PLASTICITY IN EUKARYOTES

Heredity, or transmission of qualities from ancestor to descendant, is reflected in fairly rigid chromosome organization of germ cells. In eukaryotes this may be illustrated, for instance, in genera of the same family of dicots, the Solanaceae, in the order Scrophulariales (Asteridae): the *Lycopersicon* (tomato) chromosome has a region between centromere and telomere which consists of a row of segments in which DNA is compacted into tight masses, largely inactive in transcription ('chromomeres'); in *Petunia*, in spite of being another genera of the same family, the abundance of centromeres is not preserved; as larger blocks of heterochromatin are observed [34].

These two genera of the Solanaceae Family illustrate how quickly the evolutionary process can induce rearrangements of heterochromatin, while preserving general chromosome structure. This capacity for chromosomes to be molded (their 'plasticity') preserves general chromosome organization. This property may be achieved through several mechanisms including:

3.1.1. Chromosomal mutations consisting of translocations of discrete DNA segments ('transposable elements') between non-homologous DNA sites. This process may occur not only in eukaryotes [35], but also in prokaryotes [36].

3.1.2. Horizontal gene transfer (HGT). DNA segments from one species may be transferred to another, where it may be integrated into the genome of the recipient cell. We have reviewed recently HGT, a process which may affect both eukaryotes and prokaryotes [37].

The examples mentioned in Sec. 1.2 demonstrate that some of the themes developed by eukaryotes are already present in prokaryotes, in spite of the small size of the bacterial genome. Condensation of whole chromosomes is also anticipated in the small genomes of some prokaryotes. Gene silencing has reached a central position in eukaryotic gene expression in the context of sexual reproduction [34]. However, bacterial shut-down processes in whole chromosomes have been observed in the cycle of the two developmental phases of the blindness-inducing parasite *C. trachomatics*

(cf., Sec. 1.2.) Infection of susceptible cells begins by a metabolically inert *C. trachomatics*, in which its core consists of apparently condensed chromatin [38]. Global regulation of gene expression, as exemplified by the mechanism for controlling bacterial virulence in *C. trachomatics*, is considerably developed in the much larger eukaryotic genome of metazoans and metaphytes.

3.2. ORIGINS OF FACULTATIVE HETEROCHROMATIN

Eukaryotes base their global regulation of gene expression on their ability to manipulate facultative heterochromatin. This process had to await the evolution of heterochromatin in the lower eukaryotes. The nuclei of primitive single-celled protists, such as the flagellated green alga *Clamydomonas reinhardii*, have some repeated DNA, a single nucleolus, but no heterochromatin [39].

Early work has been reported on mammalian DNA of different density composed of relatively short, highly repetitive polynucleotide sequences ('satellite DNA'.) This fraction is about 10% of all the DNA. Satellite DNA has been observed in the colourless alga *Polytoma* [40], in the euglenoid *Euglena gracilis*. The parazoan *Microcyona* (a sponge) also has satellite DNAs [39]; at this early stage in evolution in a branch separate from the metazoans, DNA may have developed repeated sequences by mechanisms analogous to gene amplification [41]. This early repetition of DNA sequences may have been preserved because they may have served some advantageous structural role. All satellite DNAs have the property of heterochromatization in common, in spite of being species-specific. Once heterochromatin had been established in higher eukaryotes the phenomenon of gene silencing was possible. In higher eukaryotes constitutive-heterochromatic, repetitive-DNA is well documented in a wide range of taxa, for instance:

3.2.1. Metazoans of diploblastic phyla (coelenterates) and triploblastic phyla (arthropods, mollusks, and chordates) [39].

3.2.2. Metaphytes from monocots of the subclass Commelindae (*Secale cereale*, rye) to dicots of the subclass Rosidae (*Phaseolus vulgaris*, bean) [39].

On the other hand, facultative heterochromatin may also be documented in a wide range of taxa:

3.2.3. In Arthropoda. The homopteran *Pseudococcus obscurus* (mealy bugs, or coccid in the Cicadidae Family) is able to silence a whole set of paternal chromosomes early in its development. In the dipteran genus *Miastor* (gall midges), it has been observed that the germ cells of these plant pathogens of the Cecidomyiidae Family have twenty-nine chromosomes, while the soma have only six. In this case 23 'E-chromosomes' are inactivated [43].

3.2.4. In Nematoda. A parasite of both vertebrates and invertebrates, *Ascaris megalocephala*, loses some chromosome segments early in its development [40].

3.2.5. In Chordata. Prototherian mammals, both marsupials and placentals, are able to silence part of their sex chromosomes, while therian mammals are able to silence the full chromosome, only leaving a few genes active [45], a point to be discussed more fully in Sec.4.
3.2.6. In Monocotiledonia. The perennial rye grass *Lolium perenne* of the Poaceae Family is characterized by two-ranked many-flowered spikelets; in this genus whole inactivated chromosomes may be concerned in the process of cell division [46]. In *S. cereale*, a species of hardy annual cereal grass, a number of supernumerary B-chromosomes are abundant in Asian populations, but are rare in Europe. These chromosomes are heterochromatic and have been shown to affect the duration of the mitotic cycle [47], as well as meiosis [48].

4. Physical aspects of eukaryotic hereditary gene regulation

4.1. PHYSICAL ASPECTS OF FACULTATIVE HETEROCHROMATIN

Mammals are probably a good taxon where we should focus attention on the physical aspects of shut-down processes of gene expression occurring in large sections, or even in whole chromosomes. The rapid evolution of this class of chordates may be exemplified, for instance, by the evolution of cetacean swimming [49]. This suggests that mammalian genomes are particularly dynamic and plastic. Indeed, mammalian genomic plasticity provides us with examples of novel physical phenomena:
4.1.1. Spread of X inactivation [50], a phenomenon which may be identified through chromosomal translocations.
4.1.2. Initiation of inactivation at a specific locus, called the X chromosome inactivation centre (XIC). This locus is identified through the expression of RNA transcripts, which are specific to the X-inactivated chromosome but remain untranslated into proteins [51].
4.1.3. Gene escape is a third phenomenon in which exceptional genes remain active. This may imply that X-inactivation is brought about by the spread of heterochromatization along the chromosome from the XIC.

The extent of the spread of X inactivation is clearly variable in ontogeny: in early embryogenesis the time of first appearance of X inactivation ranges from the blastocyst in some species to early neurulation in other species. On the other hand, late in ontogeny (in aging female mammals) inactivation may disappear [52]. It is remarkable that the extent of the spread of inactivation is also variable in phylogeny. In order to understand this property we should approach Ohno's Law that rationalizes some genetic experiments on monotremes. We first recall that this taxon, Monotremata, comprises the duck-billed platypus (*Ornithorhynchus anatinus*), as well as echidnas (*Tachyglossus* and *Zaglossus*.)

4.2 OHNO'S LAW

In his classical paper [53] Ohno postulates, as a fact, that the X chromosome of any eutherian mammal species, regardless of whether it be placental or marsupial, is the exact genetic equivalent of the human X chromosome. There is wide support for this postulate from human X-linked genes, since they are also X-linked in other mammals. Ohno's Law is supported by data from about 20 species included in several orders.

Ohno interprets this striking phenomenon as a case of a "frozen accident". Housekeeping genes such as phosphoglycerate kinase (X-linked in man, horse, and kangaroo), have no direct connexion with sex determination. Yet, they remained X-linked in other mammalian species, as the X-chromosome happened to be selected for gene silencing.

Monotremes, on the other hand, display incipient inactivation, which is observed to spread along the X-chromosome as evolutionary processes raise early mammals to the level of therian mammals. In fact, in monotremes, inactivation occurs only in the short arm of the X chromosome (X_p) in some tissues [54].

The argument for inserting these facts into the evolution of prototherian mammals is as follows: inactivation begins at an XIC. Its spreading along the chromosome is suggested by the inactivation of attached autosomal material (i.e., from chromosomes other than the sex chromosomes) in X-autosome translocations. Yet, one may argue that this autosomal inactivity could be due to a position-effect, in view of the proximity of the autosomal material concerned to X-chromosome heterochromatin.

However, both the spreading of inactivation, as well as the data from X-autosome translocations, may be compatible. As we have seen above, in Sec. 1.2, there are proteins specifically associated with position-effect variegation. These proteins could be part of the molecular mechanism for facultative heterochromatin [55]. In the course of evolution the length of the inactivated region ξ (the 'distance of spread') has increased gradually, from monotremes to eutherians [54].

5. Discussion and conclusions

5.1. PHYSICAL PARAMETERS IN X-CHROMOSOME INACTIVATION

We wish to discuss four physical parameters in relation with heterochromatization:

5.1.1. The distance of spread ξ.

5.1.2. The degree of packaging in chromatin given by the parameter $\eta = L_1 / L_2$, where L_1 denotes DNA length in the fully extended state, and L_2 denotes the length of the folded state of condensation. Heterochromatin is characterized by high values of $\eta \sim 10^4$, while the 100-angstrom DNA fiber of eukaryotes has a value of $\eta \sim 10$.

5.1.3. The rate of advancement of the replication fork through euchromatin (r_f). The interest in this parameter is justified by the fact that r_f is a variable that is subject to measurement. For instance, in yeast chromosome 3 the replication fork normally advances through euchromatin at a rate of r_f = 4kbp/min [56]. The fork, a multienzyme complex slows down by a factor of 4 as it enters the telomere, whose chromatin is in the heterochromatic state (10^4), while its spread is limited to a few kbp.

5.1.4. In first approximation late replication of heterochromatin implies the existence of a fourth parameter (λ), the direct proportionality factor between r_f and η.

Our previous analysis of this problem was based on the assumption that biological function, such as transcription, DNA replication, and compaction may be viewed as correlates of collective phenomena, rather than chemical detail [57]. This postulate received more formal bases in a preliminary analytical approach in terms of mean-field theory. Questions discussed previously included the coupling of transcription and DNA replication in eukaryotes [30], and DNA folding [58], in which the parameters r_f and η are functionally related, $r_f = (\lambda \eta)^{-1/2}$.

If the underlying idea is correct that collective effects are appropriate correlates of some biological phenomena, then certain interesting consequences may be expected: as a chromosome early in embryogenesis is active, at a certain time the XIC triggers off a signal that spreads the heterochromatic state up to a distance ξ.

In lower mammals ξ is smaller than the length of the small arm of the X chromosome $L(X_p)$, i.e., $\xi < L(X_p)$. Then, for distances $d > \xi$, the chromosome is euchromatic. From the above relationship between distance of spread and packaging density, it follows that there should be a corresponding slowing down of the replication fork in proportion to the extension of its spread of X inactivation.

We recall that a reduction in the r_f parameter has been observed in yeast. However, in prototherians a phenomenon of slowing down of the replication fork is expected to occur as this multienzyme complex enters the telomere region. According to the present analysis, there should be additional (still to be detected) increments of the r_f parameter: as the fork, starting from the centromeres, covers a distance ξ and enters the euchromatic region, the rate of fork propagation should increase.

5.2. CONCLUSIONS

We have confined our attention to eukaryogenesis, which is just one of the major evolutionary transitions that have occurred in the origin and evolution of life on Earth. Preliminary transitions may have led to the evolutionary stage immediately preceding the radiation of a eukaryotes [19]. Such transitions included a sequence of events such as chemical evolution through to the RNA world, encapsualtion

in a lipid membrane, transition of the RNA free replicators to DNA replicators, development of the genetic code and, finally, transition of free replicators to linked genes.

We have defended the thesis that the subsequent major transition, eukaryogenesis, does not have to be regarded entirely from the point of view of the effect of symbiotic incorporation of organelles [59]. To some advantage we may consider eukaryogenesis in genetic terms, mainly through the evolution of chromatin structure and function. This point of view has been referred to, in a different context, as the gene-centered approach [60].

For our purpose we have discussed possible departures from prokaryotic themes, which manifest themselves not just through larger eukaryotic genomes and richer enzymatic repertoires, but particularly through the plasticity of the eukaryotic chromosome.

Some additional difficulties that may complicate the traditional approach to eukaryogenesis include:

5.2.1. The presence of a nuclear membrane cannot be a unique signature of the eukaryotic cell, for amongst eubacteria, the planctomycete *Gemmata oscuriglobus* [61] is capable of separating their chromosomes from their ribosomes by a lipid membrane.

5.2.2. The presence of organelles as a criterion for eukaryogenesis also presents several difficulties, as some eukaryotes lack mitochondria. In fact, one phylum of protozoans, (microsporidia) consists of organisms that lack mitochondria [62]; also diplomonads are amitochondrial protists, for example *Giarda lamblia* [63]. Furthermore, chloroplasts are absent in chemotrophic protists and metazoans. Finally, it should be noticed that at least cryptomonad algae seem to have a further organelle (the nucleomorph) [64].

To sum up, in our preliminary search for the first steps towards eukaryogenesis we have provided a number of examples to support a genetic approach, which may be complementary to the insights that have been provided by cell morphology. Besides, we have attempted to focus attention on some physical aspects of chromosome plasticity that may have been prominent in the evolutionary process. We have shown that in certain cases relevant parameters can be measured, and that their relationships may be subject to analytical treatment. The present gene-centred approach leads to results that may be confronted with experiments.

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