jects—objects that are the least likely to be involved accidentally. But physically we know the least about these peculiar objects, and they are the ones for which there is the greatest a priori chance that new and unknown physical mechanisms are at work.

In the end, however, we must all agree that the ultimate criterion for science is experiment and observation. If the observational paradoxes discussed in this article can be demonstrated to be false or accidental, then we can say that the paradoxes are solved on the basis of our present knowledge. If the observations stand, then we must conclude that something new of vast importance is happening and we should get on with the exciting job of finding out more about it.

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Heterochromatin, Satellite DNA, and Cell Function

Structural DNA of eucaryotes may support and protect genes and aid in speciation.

Jorge J. Yunis and Walid G. Yasmineh

The term heterochromatin was first introduced by Heitz (*1, 2*) to denote chromosomes or chromosome regions that are condensed in interphase and prophase and do not unravel in telophase like the rest of the chromosomes. Although Heitz made his initial observation in primitive plants (liverworts and mosses), his definition of heterochromatin generally holds true for most organisms. In mammals two main types of heterochromatin are recognized: constitutive heterochromatin, or the heterochromatin that is present in homologous chromosomes, and facultative heterochromatin, or the heterochromatin that results from the inactivation of one of the two X chromosomes in females. This inactivation is an effective mechanism to reduce the number of functional X chromosomes to one in both sexes (*3*).

Recent reports indicate that the DNA of constitutive heterochromatin is composed to a large extent of short repeated polynucleotide sequences, termed satellite DNA. This discovery has necessitated a critical review of current ideas concerning the origin and function of this portion of the genome of higher organisms (*4–12*). A careful appraisal of the information that has accumulated about heterochromatin since the time of Heitz (*1, 2*) and on satellite DNA during the last decade suggests that these entities have vital structural functions: they maintain nuclear organization, protect vital regions of the genome, serve as an early pairing mechanism in meiosis, and aid in speciation.

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Satellite DNA

Satellite DNA was first detected in the early 1960's by the technique of density gradient centrifugation. When DNA of the mouse, guinea pig, calf, and crab was centrifuged in neutral CsCl, a minor component or components differed in buoyant density from the bulk of the DNA (*13–15*), and the DNA of different density was termed satellite DNA. The observation a few years later that the complimentary strands of mouse satellite DNA reassociate rapidly after denaturation by heat (*16, 17*), strongly suggested that satellite DNA is composed of relatively short, repeated polynucleotide sequences.

The relation in many organisms between repetitiveness and rate of strand reassociation was investigated soon thereafter by Britten and Kohne (*17*). They introduced the variable *Cot* (where *Co* equals the initial concentration of DNA in moles of nucleotide per liter and *t* equals the reassociation time in seconds) to estimate

redundancy within a genome (or a fraction thereof). They showed that repetitive DNA is ubiquitous among eucaryotes—from the higher protists to man—and amounts to 20 to 80 percent of the genome. In higher organisms, especially in mammals, the repetitive sequences comprise 30 to 40 percent of the total DNA and may be divided into two classes: a unique fraction of highly repetitive DNA, or satellite DNA, that (for example, in the mouse) comprises about 10 percent of the DNA and consists of polynucleotide sequences approximately 300 nucleotides long that are repeated about 1 million times, and a fraction of intermediate repetitiveness that comprises 20 percent of total DNA and consists of families of sequences repeated from 100 to 100,000 times.

As will be seen below, the repetitiveness of DNA as measured by *Cot* has been underestimated by one to two orders of magnitude because point mutations have caused mismatching of many sequences. Despite this discrepancy, however, *Cot* remains very useful in the estimation of redundancy.

Almost simultaneously with this work on the redundancy of DNA, other attributes of satellite DNA were being defined. Flamm *et al.* (*18*) showed that mouse satellite DNA is rich in adenine (A) and thymine (T) and that, when centrifuged in alkaline CsCl, this DNA separates into a heavy strand rich in T (46 percent of bases are T) and a light strand rich in A. Subsequent studies with density gradient centrifugation in CsCl, and in Cs_2SO_4 in the presence of DNA-binding cations such as Hg^{2+} or Ag^{+}, resulted in the discovery and characterization of satellite DNA's in many organisms (*8, 10, 11, 19–27*).

These studies revealed a number of important characteristics of satellite DNA's. (i) Similar to repetitive DNA, satellite DNA's occur commonly among eucaryotes (*21, 23, 27*). (ii) Satellite DNA's differ significantly from the remainder of the DNA of the genome in a number of respects; these include repetitiveness, base composition, buoyant density, binding to divalent cations, melting profiles, and separation of strands in alkaline CsCl (*8, 10, 11, 20–22, 25*). (iii) Perhaps the most remarkable feature is that satellite DNA's from different species differ in many of these respects (*5–11, 25*).

These characteristics are well illustrated in the case of the mammalian satellite DNA's (Table 1). The species studied include the mouse, guinea pig, calf, European field vole (*Microtus agrestis*), and man. Satellite DNA is about 10 percent of the DNA in these species. In each species except the mouse, more than one satellite DNA has been found; these differ from each other and from those of other species in most of the characteristics indicated.

The sequence analysis of guinea pig satellite DNA I has provided further insight into the nature of satellite DNA (*4*). Southern took advantage of the extreme difference in bouyant density of the strands of this satellite DNA to isolate the complementary strands in pure form. The strands were degraded by a mixture of diphenylamine and formic acid, and the products of this reaction were separated by two-dimensional ionophoresis. The results indicated that the basic unit is a hexamer and that the sequence of the hexamer in the light strand is C-C-C-T-A-A. Southern remarked that the original hexamer must have undergone base substitutions to yield the present-day sequence, since a significant fraction of the copies found had undergone one or more base substitutions. He also concluded that such substitutions caused an overestimation of the length of the basic unit when the length was determined from reassociation rates (see above).

A basic unit was also found in mouse satellite DNA; however, the

Table 1. Characteristics of satellite DNA; A, adenine; T, thymine; G, guanine; C, cytosine.

Type	Density in neutral CsCl (g/cm³)*	Percent in genome	T_m (°C)	(A + T)/(G + C) ratio	Strands					References
					Density in alkaline CsCl (g/cm³)	Base composition (%)				
						A	T	G	C	
				Mouse						
	1.691	10–12	86.0	1.84	1.722	44.8	20.2	20.0	13.0	(*7, 20*)
					1.742	19.2	45.8	14.0	21.4	
				Guinea pig						
I	1.705†	4–6	86.0	1.56	1.722	39.7	21.1	3.1	36.0	(*8*)
					1.800	21.8	39.6	35.7	2.9	
II	1.705†	3–5	88.0	1.30	1.722	27.7	28.2	13.5	30.6	
					1.752	29.0	27.2	31.9	11.9	
				Calf						
I	1.706†	3	85.4	1.17	1.770					(*10*)
II	1.713†	7	91.4	0.82	1.773					
					1.783					
				Micrototus agrestis						
I	1.700‡	2	76.9	200						(*11, 35*)
II	1.717‡	6	82.2	1.03						
				Man						
I	1.687§	0.5	80.0		1.707					(*21, 35*)
					1.738					
II	1.693†	2	87.0		1.740					
					1.750					
III	1.696†, ||	15	86.0							

* The density of the main DNA band was always between 1.699 and 1.700 gm/cm³. † Initially isolated in Cs_2SO_4-Ag^+. ‡ Determined after denaturation of sheared DNA, reassociation, and isolation on hydroxyapatite. § Initially isolated in Cs_2SO_4-Hg^{2+}. || This DNA, isolated by Corneo *et al.* (*21*) and termed "homogeneous," may correspond to a G + C–rich fraction isolated in our laboratory by denaturation of sheared DNA [45,000 pounds per square inch (psi) in a French pressure cell], reassociation, and fractionation on hydroxyapatite. The fraction comprises about 8 percent of the total DNA and sediments at 1.715 mg/cm³ in CsCl. After further shearing (60,000 psi) and density gradient centrifugation in CsCl, two additional subfractions sedimenting at densities of 1.725 and 1.729 g/cm³ were observed. The three subfractions show a much steeper melting profile than the original fraction sheared at 45,000 psi (*35*).

basic unit appears to be slightly longer and more complex than that characterized in the guinea pig. These studies, together with the appreciable differences often observed among satellite DNA's of closely related species (*24–26*), strongly suggest that satellite DNA does not code for protein. The observation by Flamm *et al.* (*26*) that mouse satellite DNA does not hybridize with RNA extracted from mouse liver, spleen, or kidney supports this conclusion.

Heterochromatin and Satellite DNA

An important step toward the elucidation of the origin and function of satellite DNA was the discovery that satellite DNA in higher organisms is preferentially located within certain chromosomes or chromosome segments. Schildkraut and Maio (*27*) showed in 1968 that the DNA of purified nucleoli from the mouse is enriched in satellite DNA, and we observed that constitutive heterochromatin, extracted from the liver and brain of male mice by sonication and differential centrifugation, is intimately associated with nucleoli and contains DNA that is primarily (more than 70 percent) of the satellite type (*7*). These results for constitutive heterochromatin were soon confirmed by us in the guinea pig, calf, *M. agrestis*, and man (*8–12*) (Table 1), and in the kangaroo rat and crab by other investigators (*28*).

In 1970 Jones (*29*) and Pardue and Gall (*30*) used the technique of in situ hybridization to show that mouse satellite DNA (or RNA complementary to it) hybridizes with DNA in the region of the centromere of all the metaphase chromosomes except the Y chromosome. In interphase, satellite DNA is associated with perinucleolar heterochromatin. In *M. agrestis*, RNA complementary to repetitive DNA hybridizes in situ with DNA in the regions of constitutive heterochromatin of the giant X and Y chromosomes and in small regions around the centromeres of the autosomes (*31*). The technique of in situ hybridization has also been used in lower animals, particularly those in the order Diptera, to show that satellite DNA hybridizes with the heterochromatic regions in the centromere and nucleolar organizer (secondary constriction), and in some instances with heterochromatic regions in the telomeres and with intercalary regions within the chromosomes (*32, 33*). In *Drosophila melanogaster* (*32*), the α- and β-regions of heterochromatin, which were originally described by Heitz as the dense and diffuse areas, respectively, of heterochromatin in the centromere, each hybridize with DNA of a different degree of repetitiveness.

Recently a method that uses Giemsa stain for the detection of highly repetitive (satellite) DNA within metaphase chromosomes was developed independently by Arrighi and Hsu (*34*) and Yunis and others (*12, 35, 36*). The method consists of denaturation of the DNA of metaphase chromosomes by heat or alkali and reassociation in situ under controlled conditions. For many mammals, we found that satellite DNA is preferentially located in the pericentromeric regions of all the chromosomes except the Y chromosome, in which it is mainly located in the long arms. (In man there is a very small amount of pericentromeric heterochromatin in the Y chromosome.) The animals investigated included species with large amounts of pericentromeric heterochromatin (mouse, calf, guinea pig, Syrian hamster, and horse) and species with small to minimal amounts (*Microtus californicus, M. montanus, M. ochrogaster, M. pennsylvanicus, Ellobius lutescens, M. agrestis*, Chinese hamster, and man). Reassociation was also observed in the various heterochromatic segments of the autosomes of man and Chinese hamster and in the constitutive heterochromatin of the composite sex chromosomes of Chinese hamster and *M. agrestis* (*37, 38*). Representative patterns of reassociation for mouse, guinea pig, calf, man, and *M. agrestis* are shown in Fig. 1.

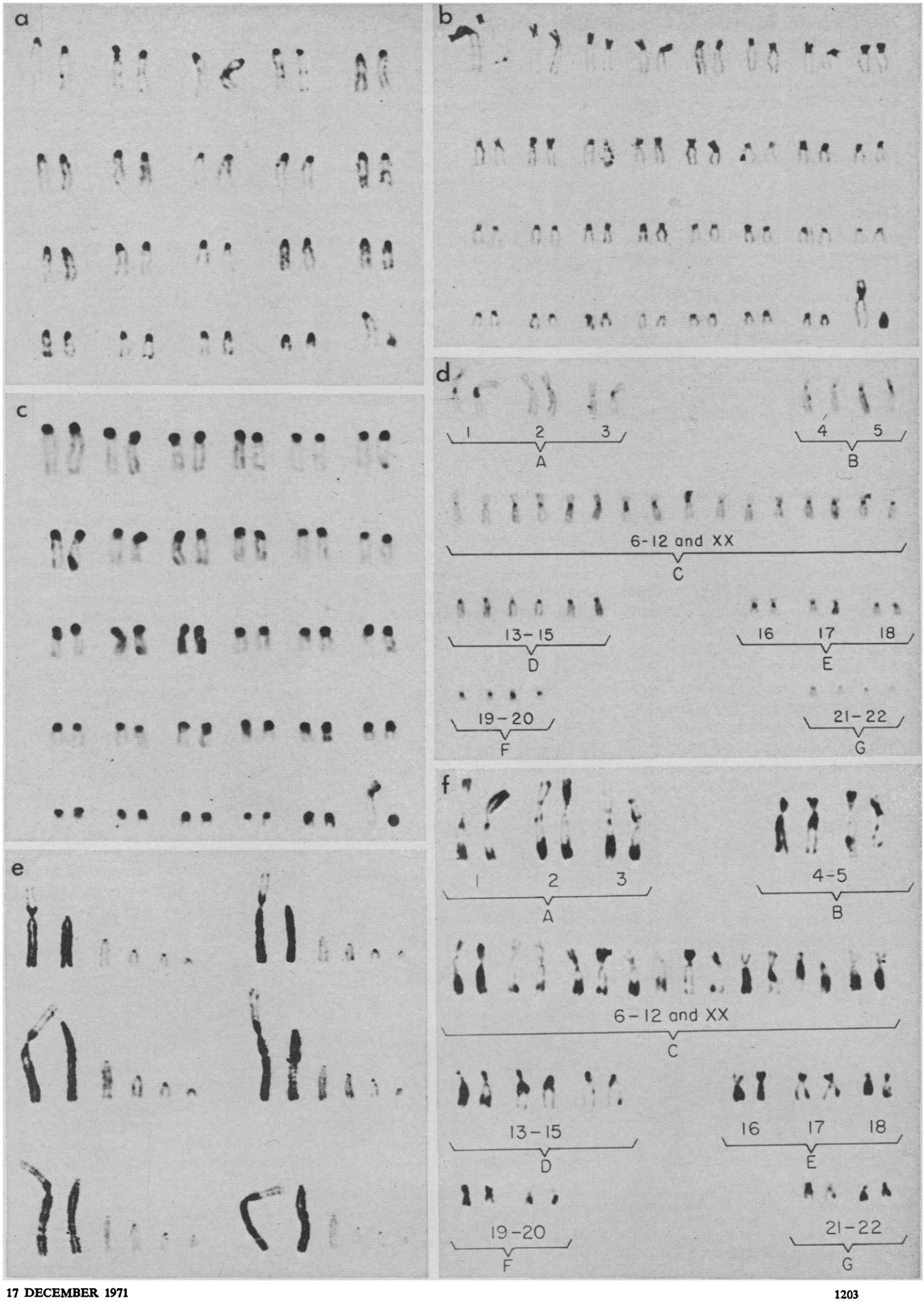
Cytological Aspects of Constitutive Heterochromatin

Early cytological studies suggested that constitutive heterochromatin is preferentially located around centromeres and nucleolar organizers and that its function may be to support the structure of these regions (*3*). Despite such observations and the paucity of genes in constitutive heterochromatin (*2, 3, 39, 40*), it was generally believed that constitutive heterochromatin is a variant state of chromatin rather than a separate entity (*3, 41*). This belief was based mainly on observations that in lower organisms, such as *D. melanogaster*, heterochromatin is not visible in early embryogenesis, and that in many organisms, including mammals, constitutive heterochromatin detected cytologically varies in amount and appearance in different adult tissues.

We have attempted to shed light on this problem by studying *M. agrestis*, because in this unique mammal most of the constitutive heterochromatin is located in the giant sex chromosomes (Fig. 1e) and can be followed easily throughout development. The results demonstrate that the constitutive heterochromatin of the giant chromosomes appears as condensed chromatin in interphase nuclei throughout the entire period of development, including the zygote and the stages of gametogenesis (*37, 42, 43*). The giant chromosomes appear as primary heterochromatic fibers, which vary in degree of folding and condensation depending upon the cell type. For example, heterochromatin appears in fibroblasts as two long and extended fibers (Fig. 2a), in hepatocytes as two large diffuse masses composed of fibers with different degrees of folding (Fig. 2b), in endothelial cells as two bands (Fig. 2c), and in neurons as two large compact masses called chromocenters (Fig. 2d). Table 2 lists the tissues that exhibit the four patterns shown in Fig. 2, a–d.

The primary fiber is seen clearly in oogonial prophase (Fig. 2e), since in oogonia only constitutive heterochromatin exhibits heteropycnosis (precocious condensation). Both X chromosomes show terminal euchromatic segments. These are seen in oogonial metaphase (Fig. 2f), where the chromatids of the X chromosomes are separated along most of the short arm but are in apposition along the remaining heterochromatic segments of

Fig. 1. Karyotypes from mouse (a), guinea pig (b), calf (c), man (d and f), and *M. agrestis* (e). The DNA was denatured in situ at 100°C and reassociated at 65°C for 30 minutes in 0.06*M* phosphate buffer, *p*H 7.0 (*12*). The reassociated satellite DNA stains darkly with Giemsa stain and corresponds closely to the chromosome segments known to contain constitutive heterochromatin (*35*). In e, partial karyotypes of six different metaphases from a male *M. agrestis* are shown. In d and f, human karyotypes are shown as they appear after 15 and 30 minutes of reassociation, respectively. [Reproduced in part (a, d, and f) from Yunis *et al.* (*12*).]


a
b
c
d
1 2 3
A
4 5
B
6-12 and XX
C
13-15
D
16 17 18
E
19-20
F
21-22
G
e
f
1 2 3
A
4-5
B
6-12 and XX
C
13-15
D
16 17 18
E
19-20
F
21-22
G

the chromosomes. In anaphase the heterochromatic segments show delayed separation of chromatids (Fig. 2g), and in telophase the giant chromosomes start to assume their original appearance in interphase (Fig. 2h).

Fiber Is Elemental Unit of Heterochromatin

Thus, in the field vole the elemental unit of constitutive heterochromatin in interphase is a fiber with varying degrees of folding, and is not the heterochromatic mass called the chromocenter. This discovery explains the failure previously to observe heterochromatin in some cell types (for example, fibroblasts and cleavage embryos). This failure occurred even for *M. agrestis* with its giant heterochromatic chromosomes (*44, 45*). Obviously, when the constitutive heterochromatin is evenly distributed among the chromosomes (as is usually the case), it would be even more difficult to detect in cells where it appears as fibers than in cells where is appears as masses.

When the distribution of constitutive heterochromatin in specific chromosomes or chromosome segments cannot be readily visualized (as is often the case), it can be deduced from the property of late replication shown by DNA of heterochromatin (*41*). For example, in the Chinese hamster, Pflueger and Yunis (*38*) observed that adult cells of ectodermal, mesodermal, and endodermal origin show the same chromosomal distribution of late-replicating DNA and therefore, presumably, of constitutive heterochromatin. In the mouse, the pericentromeric regions are heterochromatic and late-replicating in several cell types, including germ cells in meiosis, cells of cleavage embryos, and adult somatic cells (*41, 46, 47*). Using the technique of in situ reassociation of DNA in chromosomes, we observed that the DNA in these regions reassociates rapidly in cleavage embryos, in many somatic cells (fibroblasts, lymphocytes, kidney epithelial cells, and bone marrow cells), and in male germ cells in meiosis. In the hedgehog, *Erinaceus europaeus*, Gropp and Citoler (*48*) noted six large, heterochromatic, late-replicating autosomal segments in fibroblasts, lymphocytes, kidney cells, and spermatogonias.

Further evidence of the individuality of constitutive heterochromatin is derived from the observation that satellite DNA, which has been shown in several mammalian systems to form an integral part of the DNA of constitutive heterochromatin, is present to the same extent in various tissues (*13, 14*), is late-replicating (*5, 49*), and is usually located in the vicinity of the centromere and nucleolar organizer. Although the degree of condensation of constitutive heterochromatin usually varies in the different cell types (*3, 44, 50*), in some species the heterochromatin almost always appears as a compact mass (chromocenter). The chromocenter has been found in cleavage embryos (2 to 16 cells), germ cells, and various somatic tissues (including fibroblasts) of the mouse, rat, Syrian hamster, and hedgehog (*44, 46, 48, 51*).

The reason for the differences in the appearance of constitutive heterochromatin among species and cell types is still unknown. In general, the degree of condensation of constitutive heterochromatin at the molecular level may be affected by the repetitiveness of its DNA, by the preponderance of one or more bases in DNA strands (Table 1), or by a relatively high concentration of methylated minor bases (*52*). Variations among cell types in the appearance of the condensed fiber may be due to differences in the environment within the nucleus, such as variations in the concentration of divalent cations (Ca^{2+} and Mg^{2+}), polyamines, and polyanions (*53, 54*).

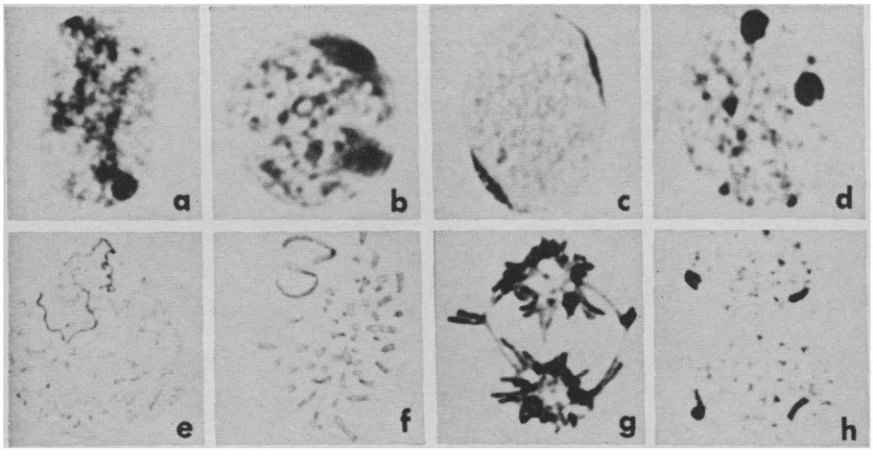


Fig. 2. Representative patterns of the giant sex chromosomes of *M. agrestis* in interphase and dividing nuclei. Shown are interphase nuclei from a fibroblast (a), hepatocyte (b), endothelial cell (c), and neuron (d); prophase (e) and metaphase (f) nuclei from oogonias; and anaphase (g) and telophase (h) nuclei from spermatogonias. [Reproduced from Lee and Yunis (*37, 42*)]

Structural Role of Constitutive Heterochromatin

Constitutive heterochromatin may be considered a special type of chromatin that contains most of the satellite DNA, that is, the highly repetitive DNA sequences of the genome that are not transcribed into RNA for protein synthesis. Blocks of these sequences are usually located in the regions of the nucleolar organizer, centromeres, and telomeres, or are sometimes intercalated within other regions of the chromosomes. An evaluation of the knowledge about constitutive heterochromatin and satellite DNA suggests that the roles of constitutive heterochromatin are structural in nature. (i) It may protect vital areas of the genome from external disruptive forces and evolutionary change. (ii) It may attract homologous chromosomes for initial alignment during meiosis and nonhomologous chromosomes for establishing proximity between chromosomes or chromosome regions that are functionally related. (iii) It may establish "fertility barriers" that provide means for evolutionary diversity and speciation.

Around the nucleolar organizer, heterochromatin (or satellite DNA) may serve as a spacer to protect the cistrons for 18*S* and 28*S* ribosomal RNA from mutation and crossover. Such protection is essential in view of the amazing conservation throughout evolution of these cistrons, which are linked and repeated in tandem (*55*). Heterochromatin is closely associated with nucleoli in both plants and animals (*3, 35, 39*). Although such association has sometimes been ignored, recent electron microscopic studies (*56*), judged in the light of the association of satellite DNA with the

nucleolar organizer (*27, 29, 30*), strongly suggest that there is a continuum between perinucleolar heterochromatin and intranucleolar chromatin fibers.

In the oocytes of some lower animals (echinoderms, insects, and amphibians), cistrons for ribosomal RNA are repeated extensively. The amount of satellite DNA is often increased in a proportionate manner. The satellite DNA spaces individual cistrons (*41, 57–59*) or is somehow associated with a large block of cistrons in the form of a large heterochromatic body (*41*). Since this DNA is not transcribed and shows marked quantitative and qualitative differences even among closely related species (*58*), its function may be to protect the ribosomal RNA cistrons. There is strong evidence that crossing-over in plants and animals is less frequent in heterochromatin than in euchromatin. Condensed regions may attract homologous chromosomes at the onset of meiosis, but these regions probably prevent the intimate pairing that is necessary for crossing-over (*60–65*). Constitutive heterochromatin may thus preserve polycistronic genes (such as those of transfer RNA and *5S* ribosomal RNA), and conceivably single genes that have withstood evolutionary change, from crossing-over and mutation.

The universal presence of constitutive heterochromatin around the centromeric region of chromosomes is well established (*3, 39, 66, 67*). Pericentromeric heterochromatin may provide centromeric "strength"—that is, it may ensure that proper separation of chromosomes occurs during cell division (*68*). This idea, originally formulated by Novitsky for *D. melanogaster*, has been recently emphasized by Brown (*3*) and by Walker (*5*). Heterochromatin, which appears to protect the nucleolar organizer, may also protect the centromeric region, which provides attachment points for the spindle fiber proteins. Spindle fiber proteins, like the ribosomal RNA's, have probably undergone little evolutionary change (*55*).

Heterochromatin Brings Chromosomes Together

The aggregation of chromosomes through heterochromatin was noted in the early 1930's by Heitz (*69*), who showed that the polytene chromosomes of the salivary glands of *D. melanogaster* are joined through centromeric heterochromatin to form one large chromocenter. Since that time it has been recognized that heterochromatin in general forms aggregates between both homologous and nonhomologous chromosomes during the mitotic and meiotic cycles. Perhaps the best known example of the aggregation of nonhomologous chromosomes is the proximity during most of the cell cycle between heterochromatic regions bearing nucleolar organizers. Such aggregations are frequently observed in plants and mammals (*39, 70–72*). In man, the acrocentric chromosomes D (13 to 15) and G (21 to 22), which bear nucleolar organizers, are frequently observed to be close to each other during mitosis and meiosis (*71, 72*). These chromosomes are also frequently observed to be in proximity to chromosomes 1, 2, 18, and a chromosome in group C (6 to 12), all of which carry secondary constrictions other than those of the nucleolar organizer (*71*). The significance of these constrictions is as yet unknown, but they may represent the sites for the large number of cistrons for *5S* RNA recently discovered in eucaryotes. In this regard, Wimber and Steffensen (*73*) observed that in polytene chromosomes of *Drosophila*, region 57L of chromosome II (where these RNA cistrons are located) is frequently found in proximity to the nucleolar organizer region in the X chromosome. In man, these constrictions are within regions that are late-replicating and heterochromatic (*35, 74*), as will be seen below.

Table 2. Patterns of constitutive heterochromation of the giant chromosomes in interphase nuclei of *Microtus agrestis* [from Lee and Yunis (*42*)].

Long extended fibers
Cleavage embryo
Skin (epidermis, fibroblast)
Kidney (proximal tubules)
Lens (epithelium)
Ovary (ovum, granulosa, lutein cells)
Testis (Sertoli cell)
Large diffuse masses
Adrenal (cortex, medulla)
Cartilage (perichondrium)
Esophagus (epithelium, smooth muscle)
Fallopian tube (epithelium)
Heart (myocardium)
Intestine (epithelium, smooth muscle)
Liver (hepatocyte)
Lung (bronchiole)
Ovary (follicular cell, germinal epithelium, thecal cell)
Pancreas (duct epithelium)
Salivary gland (acini, striated duct and intercalated duct epithelium)
Skeletal muscle
Skin (sweat gland)
Trachea (epithelium, gland cell)
Ureter (epithelium)
Uterus (myometrium, endometrium, squamous and columnar cervical epithelium)
Vas deferens (epithelium, smooth muscle)
Compact bands
Blood vessel (endothelium)
Testis (lining cells of tubules)
Large compact masses
Bone (osteocyte)
Cartilage (chondrocyte)
Cerebellum (all nerve cell types)
Cerebrum (all nerve cell types)
Kidney (glomerulus, distal and collecting tubules)
Intestine (Auerbach plexus)
Lung (alveoli)
Ovary (oogonia, interstitial cell)
Pancreas (acini, islet)
Parathyroid (chief cell)
Retina (inner nuclear layer)
Skin (mast cell)
Spinal cord (nerve cell, ependymal cell)
Testis (spermatogonia, spermatocyte, Leydig cell)
Thyroid (follicle)

Aggregation of nonhomologous chromosomes through pericentromeric or telomeric heterochromatin is also frequently observed in both gonial and somatic cells (*39, 67, 75*). In the somatic cells of many species, heterochromatic regions have a tendency to clump together to form relatively large chromocenters, as in the salivary chromosomes of the order Diptera. In certain plants (*Allium cepa, Crepis capillaries*), such aggregations result in the grouping of centromeres near one pole of the nucleus and the telomeres near the other (*66, 67, 76*). In mammals, where centromeric heterochromatin is most commonly present, the presence or absence of aggregation is dependent upon the cell type (*44*). Such differences between species may be a reflection of differences in chromosomal organization at various phylogenetic levels. In germ cells, aggregation of nonhomologous centromeric and telomeric heterochromatin is observed in premeiotic interphase or early meiotic prophase and is exemplified by the well-known bouquet formation (*39, 75*). This striking aggregation of heterochromatic regions appears to be universal in occurrence, having been observed even in fungi (*60, 75, 77*). Since the bouquet arrangement brings together centromeric and telomeric regions of chromosomes before meiotic pairing, this arrangement may also bring together heterochromatic regions of homologous chromosomes and permit the alignment of long regions of homologs before true pairing begins.

The best evidence for the initial pairing of homologous chromosomes through heterochromatin comes from various plants (*Zea mays, Fritillaria lanceolata, Salvia nemorosa,* and *Impatiens balsamina*) in which homologous

heterochromatic chromosomes or chromosome regions can be shown to be paired in germ cells at the onset of meiosis and in somatic cells during interphase (*62, 78*). It has been proposed by Westergaard and others (*76, 79*) that such pairing is a remnant of a mechanism of somatic pairing and crossing-over that orginated in primitive organisms prior to the emergence of sexuality, and that this mechanism is an important initial step in synapsis during meiosis in these species. In *Plantago ovata*, the four pairs of chromosomes have large centric blocks of heterochromatin, and the homologous heterochromatic regions are believed to be paired in premeiotic interphase but not in mitotic (somatic) interphase (*60*). Using the Giemsa stain for satellite DNA, we have also observed that in early meiotic prophase (zygonema) in male mice, heterochromatic blocks are arranged in pairs; this suggests an early attraction of homologous chromosomes through heterochromatin (*35*).

Consequences of Heterochromatin Pairing

If the idea of aggregations between homologous and nonhomologous chromosomes through heterochromatin (satellite DNA) is correct, certain interesting consequences may be expected. (i) Premeiotic pairing through heterochromatin would impose limitations on the number of mutations that can be sustained by the satellite DNA of heterochromatin without loss of the ability for alignment. (ii) The species specificity of satellite DNA may provide "fertility barriers" that would prevent initial pairing of heterochromatin from closely related species. (iii) Because of the apparent susceptibility of the DNA of heterochromatin to mutation (*6, 80*) (probably by virtue of its repetitive nature and structural function), strains of a given species which have been geographically separated for many years may develop "fertility barriers," since cross-fertilization may result in atypical association of chromosomes.

In this regard, the phenomenon of genetic "affinity" described by Michie (*81*) and Wallace (*82*) can be explained on the basis of mutational changes accumulated in satellite DNA. Wallace observed that "in murine subspecific hybrids there is a tendency for centromeres of [the same] ancestral origin to segregate in meiosis to the same pole of the cell resulting in apparent linkages between unlinked loci." A similar tendency was observed in hybrids between tobacco and laboratory mice (*83*), animals whose satellite DNA's have different buoyant densities in CsCl (*84*). By the same token the picturesque chromosome patterns often observed in plant hybrids (*54*) and the dramatic behavior of chromosomes in heterokaryons of mammals (*85*) may well represent examples of mild and severe heterochromatin incompatibility, respectively.

Another important role for constitutive heterochromatin in speciation was postulated in 1937 by Darlington (*63*), who observed that heterochromatin around the centromere of acrocentric chromosomes facilitates the evolution of karyotypes by allowing viable chromosome translocations to occur. Such translocations frequently result in the loss of heterochromatin and centromeres. Swanson (*39*) suggested that "a certain amount of heterochromatin is evolutionarily desirable as a safety factor permitting a greater degree of variability than an inflexible system composed of euchromatin alone." For example, Matthey (*86*) and others (*44, 87*) observed for mammals that the subfamilies of the rodents Muridae, Cricetidae, and Microtinae, which have a large amount of pericentromeric heterochromatin, show a wide diversity of karyotypes and species, while the Felidae (Carnivora), which have relatively small amounts of heterochromatin, comprise only a few species of similar karyotype. Observations of this type have been strengthened by experiments with mutagenic agents (x-rays and alkylating agents), which showed that viable chromosomal rearrangements occur primarily at the expense of heterochromatin (*80, 88*).

Heterochromatin Content, Congenital Defects, and Neoplasia

Although heterochromatin is widespread in nature and may be present in approximately the same amount within certain phylogenetic levels, changes in heterochromatin content may occur if they are advantageous to the organism (as in the case of Robertsonian translocations) and do not impair the functioning of heterochromatin or result in the loss of essential genes. The loss or gain of constitutive heterochromatin with apparently no phenotypic effect is sometimes observed as a polymorphic trait. In the guinea pig, for example, polymorphism of the heterochromatic short arm of chromosome 1 (the short arm is either absent or present in double the amount) occurs as a normal trait without apparent phenotypic effect (*89*). In man, we have recently observed a common polymorphism of the large segments of pericentromeric heterochromatin in chromosomes 1, C9, and 16 (Fig. 1d) and in the heterochromatic long arm of the Y chromosome, all of which may be reduced in size without affecting phenotype (*35*). These heterochromatic regions have pronounced secondary constrictions that may represent sites of polycistronic genes such as those of *5S* RNA.

Under exceptional circumstances species may do well with very small amounts of heterochromatin. This is illustrated in the case of members of the subfamily of rodents Microtinae, some of which contain an amount of heterochromatin that is comparable to that in most mammals (*M. agrestis*) while others contain very small amounts (*M. pennsylvanicus* and *Ellobius lutescens*) (*44, 90*). Recent observations by us indicate that such reductions occur mainly at the expense of highly repetitive DNA ($Cot = 2 \times 10^{-5}$), which amounts to approximately 8 percent of the DNA in *M. agrestis*, 2 percent of that in *M. pennsylvanicus*, and 1.5 percent of that in *E. lutescens*. However, these small amounts of heterochromatin (or highly repetitive DNA) are usually located around the vital regions of the centromere and nucleolar organizer (*35*).

A special case of variation in the amount of heterochromatin is represented by the supernumerary B chromosomes in some plants and insects (*39*). These chromosomes are usually late-replicating (*91*) and incapable of forming chiasma (*3, 39*), and in the grasshopper they are enriched in satellite DNA (*92*). Their number in any one species varies widely with ecological conditions. This variability allows for rapid adaptability to changes in the environment, although the manner by which this is accomplished is not known. In rye, maize, and grasshoppers an increase in the number of B chromosomes is associated with an increase in the rate of crossing-over in the regular chromosomes (*65, 93*). This suggests that the B chromosomes confer some type of diversity upon off-

spring which allows them to cope with changes in the environment.

In certain lower animals, a peculiar loss of heterochromatic chromosomes or chromosome segments occurs during early development. This phenomenon is particularly striking in *Ascaris megalocephala* (*94*), in which the heterochromatic ends of the chromosomes are present in the germ cells and early cleavage embryos but are expelled from somatic cell nuclei early in embryogenesis. The repetitive nature of these chromosome segments and the possibility that genes (ribosomal?) are included in the segments remains to be explored.

Phenotypic Effects

Still another kind of variation in the amount per cell of constitutive heterochromatin is found in some species in which some individuals have less or more heterochromatin than is normal. These changes produce phenotypic effects of varying severity. In *D. melanogaster*, for example, heterozygotes for a deletion of the nucleolar organizer region have only 50 percent of the normal number of cistrons for 18*S* and 28*S* ribosomal RNA but show no conspicuous phenotypic effects (*95*), while deletion in one of the Minute regions, in which the transfer RNA cistrons are believed to be located, produces severe but nonspecific generalized effects early in embryogenesis (*96*).

Similarly in man, in most of the translocations involving two group G chromosomes, two group D chromosomes, or one from each group, a centromere and one of ten nucleolar organizers are lost without deleterious effects (*97*). On the other hand, for the viable defects of autosomal chromosomes (trisomies, partial trisomies, partial deletions), which involve heterochromatic chromosomes or chromosome segments (*97*), symptoms are largely nonspecific and show wide variation and overlap among patients with the same autosomal aberration. Usually symptoms are physical and mental retardation and gross malformations of the extremities and internal organs. The most distinct effect of these chromosome defects appears to be interference in the early development of many organs. There is a decrease in the number of cells per organ, delayed disappearance of fetal characteristics after birth, and general disarrangement of cell metabolism (*98*). This picture is compatible with the proposal that constitutive heterochromatin is mainly nontranscriptional but contains cistrons which are involved in translation (such as those of transfer RNA) and the regulation of gene expression.

A finding that may possibly link heterochromatin with the development of virus-induced tumors is the recent observation by Smith (*99*) that polyoma infection of mouse fibroblasts in culture causes satellite DNA to replicate at the beginning instead at the end of the DNA period of synthesis. More recent studies in cell lines derived from the African green monkey also suggest that the presence or absence of heavy satellite DNA may determine the response to infection by SV40 virus (*100*). Extension of such work to transformed cell lines and other oncogenic viruses may provide much-needed information concerning the mode of action of these viruses in inducing neoplasia. It is possible that some oncogenic viruses become inserted in the heterochromatic regions (satellite DNA) and cause them to replicate early, thereby upsetting chromosomal organization and function. It should be stressed here that the bulk of the aberrations in chromosome structure and number commonly observed in other types of neoplasia may also involve heterochromatin because of its great susceptibility to breakage by mutagens, its repetitive nature, and its tendency to form aggregates during the cell cycle (*80*).

Evolution of Satellite DNA and Constitutive Heterochromatin

The elucidation of the relation between satellite DNA and heterochromatin, as detailed above, permits some conjecture concerning the origin and function of this significant portion of the genome of higher organisms. Satellite DNA (or constitutive heterochromatin) possibly evolved in eucaryotes in response to an increase in genome size and in the complexity of chromosomal and nuclear organization of the cell. In procaryotes (bacteria, blue-green algae), where the need for chromosomal and nuclear organization is minimal because the genome is small and simple, neither heterochromatin nor satellite DNA is present (*17, 54, 101*). The bacterial cell, for example, has only a few cistrons for ribosomal RNA (*102*) and seems to have achieved nuclear stability by incorporating its genome in one circular chromosome in which DNA replication is initiated at one point and terminated at another.

During the evolution of the higher protists (algae, protozoa, fungi, and slime molds), the size of the genome increased. This probably necessitated the compartmentalization of the genome into more than one chromosome, the presence of multiple initiation points for replication (*54, 103*), the development of the nucleolus, centromere, and heterochromatin, and the emergence of repetitive DNA sequences with a structural function. It is not known when or in what order these events occurred. A rudimentary nucleolus and some repetitive DNA probably evolved prior to the emergence of the centromere and heterochromatin because the nuclei of primitive unicellular eucaryotes, such as the flagellated green algae *Chlamydomonas reinhardii,* possess a single nucleolus and repeated DNA sequences but their chromosomes are devoid of kinetochores and heterochromatin (*17, 54, 104*). Centromeres and constitutive heterochromatin have been found by McClintock in the fungus *Neurospora crassa* (*105*) and by others in the brown algae *Halidrys siliquosa* (*106*) and in the multicellular green algae *Nitella missouriensis* (*107*).

The earliest organisms in evolution to contain satellite DNA appear also to be the higher protists, although the presence of satellite DNA has not been correlated with that of heterochromatin. A satellite DNA that displays the characteristics of that found in heterochromatin of higher organisms has been recently observed in the colorless algae *Polytoma* (*108*). At such an early stage in evolution, organisms possessing highly repetitive DNA may have evolved by a phenomenon similar to that of gene amplification (repetition) in lower organisms, except that the repeated sequence was too small to code for protein (*5, 6, 41, 59, 109*). The repeated sequences were kept by natural selection and incorporated in the genome because of their usefulness as purely structural components that serve, for example, as spacers for vital regions that cannot sustain evolutionary change.

Furthermore, satellite DNA could probably tolerate more mutations than euchromatin because many more mutations would be needed to impair its structural role. Therefore satellite DNA was more apt to degenerate and consequently require replacement. Such a

fast turnover of satellite DNA probably resulted in the species specificity presently observed in most organisms. The specificity may simply be due to random choice of the short segment that is duplicated. The base distributions possible in such a short segment must be quite limited and would explain the strand bias (difference in base composition between strands) frequently observed in satellites. For example, a segment six nucleotides long (such as that of guinea pig satellite DNA I) cannot possibly contain the four bases evenly distributed.

Mechanism for Appearance of Satellite DNA

Thus, if the species specificity of satellite DNA proves to be general, a mechanism for the relatively rapid appearance of satellite DNA probably exists. The mechanism may be one in which a small segment of pericentromeric or perinucleolar DNA is amplified (copied) to a great extent in meiosis, with the amplified segment originating in one chromosome and spreading to others due to the proximity of centromeres and nucleolar organizers. Although the mechanism of such spread is not known, it is especially evident in the case of the mouse where Jones (*29*) and Pardue and Gall (*30*) found by in situ hybridization that the satellite DNA is evenly distributed in the centromeric chromatin of all the autosomes and the X chromosome. Satellite DNA was not found in the centromeric region of the Y chromosome, although we have recently shown by the use of in situ reassociation that the long arm contains highly repetitive DNA (*12*) (Fig. 1a).

The presence of mouse satellite DNA in the centromeric region of the X chromosome and its absence from that of the Y chromosome is of particular interest because of the observation by Ohno (*55, 110*) that the mammalian sex chromosomes originated from a common ancestral pair of chromosomes and that subsequently the X chromosome was preserved in toto while the Y chromosome underwent drastic changes. In view of this and the fact that mouse satellite DNA is different from that of other mammals (*25, 26*) it would appear that (i) satellite DNA originated with or after the emergence of the mouse as a new species and (ii) the satellite DNA of the X chromosome probably originated in an autosome, from which it then spread to all other chromosomes except the Y chromosome. The mechanism of distribution of the satellite DNA would be greatly simplified if some kind of interconnection is established between centromeres. Such interconnections are consistent with the tendency of centromeres to associate at the onset of meiosis. In a recent review DuPraw (*54*) made a special point of such associations in relation to what he termed "suprachromosomal organization."

The postulation of the rapid emergence of new satellite DNA's to explain their species specificity necessitates an explanation of how the old satellite DNA's were lost. It is conceivable that these might have accumulated a large number of mutations and that some were lost through chromosomal breakage and rearrangement, while others became intercalated in the genome and formed a large portion of the poorly studied sequences termed intermediate repetitive DNA. The species specificity of satellite DNA and the observation by Southern (*4*) that the duplicated unit of satellite DNA is much smaller than originally estimated from reassociation measurements render unlikely the possibility that these sequences evolved into DNA's that can be translated into protein (*6*). Rather, it would appear that any sequences that originated in satellite DNA would still have roles which are structural in nature. Some might have undergone few mutations but became intercalated in small blocks in euchromatin [as suggested by the work of Flamm *et al.* (*26*) in the mouse], others might have undergone many mutations and were held in heterochromatin or became intercalated in euchromatin. Within euchromatin, these sequences may serve as transcriptional stops, initiation sites for polymerases, sites of attachments to the nuclear membrane, pairing and crossing-over sites in meiosis, or as folding sites to maintain chromosome structure during the cell cycle (*35*). Since it has been estimated that no more than 10 percent of the DNA of the mammalian genome codes for protein (*111*), it is possible that the bulk of the remaining DNA is composed of these mutated sequences of satellite DNA. Perhaps the best approach to investigate this problem is to examine the DNA of constitutive heterochromatin for any differences in repetitiveness which are caused by mutated satellite DNA sequences.

Summary

With the assumption that a portion that comprises some 10 percent of the genomes in higher organisms cannot be without a raison d'être, an extensive review led us to conclude that a certain amount of constitutive heterochromatin is essential in multicellular organisms at two levels of organization, chromosomal and nuclear. At the chromosomal level, constitutive heterochromatin is present around vital areas within the chromosomes. Around the centromeres, for example, heterochromatin is believed to confer protection and strength to the centromeric chromatin. Around secondary constrictions, heterochromatic blocks may ensure against evolutionary change of ribosomal cistrons by decreasing the frequency of crossing-over in these cistrons in meiosis and absorbing the effects of mutagenic agents. During meiosis heterochromatin may aid in the initial alignment of chromosomes prior to synapsis and may facilitate speciation by allowing chromosomal rearrangement and providing, through the species specificity of its DNA, barriers against cross-fertilization.

At the nuclear level of organization, constitutive heterochromatin may help maintain the proper spatial relationships necessary for the efficient operation of the cell through the stages of mitosis and meiosis. In the unicellular procaryotes, the presence of a small amount of genetic information in one chromosome obviates the need for constitutive heterochromatin and a nuclear membrane. At higher levels of organization, with an increase in the size of the genome and with evolution of cellular and sexual differentiation, the need for compartmentalization and structural components in the nucleus became imminent. The portion of the genome that was concerned with synthesis of ribosomal RNA was enlarged and localized in specific chromosomes, and the centromere became part of each chromosome when the mitotic spindle was developed in evolution. Concomitant with these changes in the genome, repetitive sequences in the form of constitutive heterochromatin appeared, probably as a result of large-scale duplication. The repetitive DNA's were kept through natural selection because of their importance in preserving these vital regions and in maintaining the structural and functional integrity of the nucleus.

The association of satellite (or highly repetitive) DNA with constitutive het-

erochromatin is understandable, since it stresses the importance of the structural rather than transcriptional roles of these entities. Nuclear satellite DNA's have one property in common despite their species specificity, namely heterochromatization. In this sense the apparent species specificity of satellite DNA may be the result of natural selection for duplicated short polynucleotide segments that are nontranscriptional and can be utilized in specific structural roles.

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