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Cambridge
Scholars
Publishing

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This book first published 2020

Cambridge Scholars Publishing

Lady Stephenson Library, Newcastle upon Tyne, NE6 2PA, UK

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN (10): 1-5275-5975-0
ISBN (13): 978-1-5275-5975-2

To my parents, wife, and sons

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ABSTRACT

The results of nearly 50 years of study into human chromosomal Q-heterochromatin regions (Q-HRs) are summed up in this book. Research in this area has not been systematized since the 1980s. The results of this research show that this type of constitutive heterochromatin is found only in the genome of the three higher primates (*Homo sapiens*, *Pan troglodytes*, and *Gorilla gorilla*). It has also been found that: a) there is wide variability in the amount of chromosomal Q-HRs, which are primarily inherent in human populations; b) interpopulation differences are undoubtedly related to environmental factors, rather than to racial or ethnic features. This detailed account presents the history of the problem, the phenomenon of differential fluorescence of chromosomes, and the methodological approaches used in their study. Data are also presented on the distribution of chromosomal Q-HRs in relation to sex, age, norm, and pathology. The bulk of existing data on human chromosomal Q-HRs testify that they constitute a self-sustaining genetic system subject to natural selection and, together with C-HRs, play a part in cell thermoregulation, depending upon the amount present in the genome of a given individual, with all its ensuing consequences.

PREFACE

Supporters of Darwinism have gathered convincing evidence that primates originated about 70 million years ago when the global climate was warmer and the current continents had a different location: North America was connected to Europe and Asia, while South America and Africa were separated from North America by a wide sea. Further evolution of primates took place against the background of serious climatic and geographical changes, for example, the global climate has gradually become cooler, especially during the winter seasons. Forest areas began to decrease, giving way to open treeless savannas and areas covered with ice appeared on the surface of the planet.

Especially dramatic changes in the global climate have occurred in the last 2-2.5 million years and these changes coincided with the appearance of the first hominids. Science does not yet know whether the emergence of the first *Homo species* was the result of profound climate change on the African continent, or whether cold weather and other physical environmental factors contributed to the emergence of some species and the extinction of others, thereby accelerating the evolution of all mammals. Since very few species of *Homo* have survived up to the present day, it is difficult to develop a full picture predicting events in the near future, including at the genome level.

It is only known that fluctuations in climate in the last 2.5 million years have had a strong impact on all organisms and ecosystems and hominids are no exception. Be that as it may, the evolutionary success of our ancestors during this period is evidence of their high plasticity, first in Africa and then in the face of the new, even more severe, natural conditions of Eurasia. However, this test was passed by only one species (*Homo sapiens*). The remaining surviving primates (about 230 species) remained in the tropical and subtropical provinces of Africa, South America, India, and Southeast Asia.

Most people believe that man was always set to occupy the dominant position on Earth—the place that he now occupies. However, evolutionary biologists believe that even the very origin of man was not an inevitable event and he has repeatedly been on the verge of extinction. Our knowledge of human biology and paleoanthropology shows that 20,000-30,000 years ago we were not even outwardly as different as we are now. Most of these external anthropological (racial) differences are considered to be the result

of adaptive human evolution to different climatogeographic conditions and some of them appeared after some of our ancestors left Africa and settled Eurasia and Oceania. We are now more than seven billion in number and we have inhabited almost all the lands of the Earth, including the Far North, high-altitude areas, and hot deserts. Some 12,000 years ago, about ten million people lived on the Earth.

About a century and a half ago, Thomas Huxley, a follower and contemporary of Charles Darwin, compared the anatomical features of man with other primates and showed that there are no fundamental differences between them. His task was to establish unity between man and the animal world. The results of comparative studies conducted in recent decades at the molecular and chromosomal level have fully confirmed the correctness of Darwin and Huxley. Moreover, these data suggest that the differences between humans and chimpanzees are even slightly smaller than those between chimpanzees and gorillas. The results of studies on the hybridization of DNA between humans and chimpanzees show a commonality of almost 98.7 %—a value that allows the possibility of a viable hybrid.

The totality of the existing data shows that all modern humans are descended from one ancestral group of hominids who lived about 100,000-130,000 years ago in East Africa, and not millions of years ago as once assumed. 30,000-50,000 years ago, some of them managed to leave Africa and expanded to inhabit the whole of the Earth. However, how they managed to do so, while remaining a single tropical species, is a subject of long-standing and heated debate.

It is believed that the uniqueness of adaptive human evolution consisted of a combination of three components: high physiological plasticity, cultural evolution, and natural selection. If this is the case, then where, from what, and when did humans first appear. Man has high physiological plasticity and, most importantly, a high-functioning mind, without which there would be no cultural evolution. The question remains as to whether these factors are genetic in nature.

As is well known, man does not have a single protein or enzyme that has no analogue in the world of other living beings. However, in another respect the human genome is unique: among the animals and plants studied to date, only the genome of the three highest primates—human, chimpanzees, and gorillas—contain Q-heterochromatin. As it turns out, unlike chimpanzees and gorillas, only humans display a wide hereditary variability, the detailed description of which is the subject of this work.

After the collapse of the USSR, our laboratory received financial support in 1993 and 1995 from the International Science Foundation, USA and DIFCO Laboratories Ltd., Great Britain, for which we offer many thanks.

I express my sincere thanks to my colleagues who worked with me over the years in the Laboratory of Human Genetics at the National Center of Cardiology and Internal Medicine of the Kyrgyz Republic, including E.I. Aksenrod, S. Zhumaliev, G.U. Kurmanova, S.A. Nazarenko, G.O. Karagulova and S. Tabaldiev. Quantitative analysis and mathematical modeling were performed with the participation of E.Kh. Ginzburg (Institute of Cytology and Genetics, RAS, Novosibirsk) and V.V. Popov (Institute of Physics and Mathematics of the NAS of the Kyrgyz Republic). Although the title page of this book does not have their last names, they are as much authors of this work as I. I am responsible for the evaluation of data from other authors, without whom this monograph would not exist and, of course, for any errors.

Bishkek, 2020

PART I

THE PROBLEM

"Nothing in Biology Makes Sense Except in the Light of Evolution".
—Th. Dobzhansky (American Biology Teacher, 1973, 35: 125-129).

Over five million years of evolution, separating man from his closest kinsmen (the chimpanzee (*Pan troglodytes*) and the gorilla (*Gorilla gorilla*)), he has changed a lot: he has lost his fur, became completely upright, learned how to make tools, mastered speech, and created culture and science. Humans now number more than seven billion and we have settled, practically, the entire land area of the Earth, including the circumpolar belt, high altitude areas, and deserts (some 12,000 years ago, about ten million people lived on the Earth). All this happened relatively recently, as man only went beyond East Africa 30,000-50,000 years ago.

Inevitably, the question arises: why did all this happen? The answer to this question, it would seem, is that humans developed reason and turned north, while some of them reached Patagonia through Beringia. Few people doubt that man has succeeded thanks to his ability to maintain the tropical microenvironment around his body ("tropicalization") in the form of clothing and buildings. Prior to this, our distant ancestors lived where our closest evolutionary relatives (the chimpanzee and gorilla) remain today.

If this process is connected to the capacity of the human mind, then how did man become the owner of such a powerful advantage? What changes occurred in his body? Perhaps during this time, we acquired some unique genes or complexes of genes that even our closest relatives in evolutionary history, the chimpanzee, does not have? Molecular biology data suggests that the differences between humans and chimpanzees are even slightly smaller than the differences between chimpanzees and gorillas and the results of studies on the hybridization of DNA between humans and chimpanzees shows a commonality of almost 98.7%—a value that allows for a viable hybrid.

Perhaps the methods of modern biology are insufficient to understand it? Far from it. As such, why has only one species out of the 230 in the

primate family had such an obvious advantage? Why did other mammals who arose long before primates and whose descendants exist in Africa or another continent not acquire such a gene(s)? Common sense and scientific experience accumulated since the time of Lamarck and Darwin suggest that the human genome should still have some features that, along with the cultural heritage accumulated by human history, provide for the dominant position of *Homo sapiens sapiens* on Earth.

However, the true nature of such features, including genetic ones, remains unknown. Since people from different parts of the Earth or various racial-ethnic groups produce quite normal and viable descendants, there are no serious reasons to believe that significant genetic changes have occurred in the human genome during their adaptation to different climatic and geographical provinces.

On the other hand, it is known that between individuals belonging to different racial/ethnic groups, there are indeed inherited external differences, as evidenced by children born from interethnic marriages. Since these inherited anthropological differences (races) have arisen in the last 20,000 years, and this period coincides with the history of modern human development of the most diverse climatic and geographical provinces of the Earth, we have to admit that these external differences seem to be related to this adaptive evolution.

It is considered that man was initially well adapted to a hot and dry climate. This consideration is based on two arguments: 1) man as a species was formed in the tropical and subtropical climates of Africa; 2) man has very effective physiological mechanisms to combat the overheating of his body, like sweating. To all this, man, depending on the specifics of his permanent place of residence and lifestyle, has developed many behavioral and cultural reactions. Since many other animals (e.g. the camel) are adapted to a hot and dry climate, and sometimes even better adapted than humans, it is not clear why this should be: were the same genes (or complexes of genes) inherent in all mammals used or did the adaptive evolution of man occur in a completely different fashion?

In the north, permanent human sites appeared long before the appearance of the first civilizations. Nevertheless, modern man does not well tolerate the effects of cold and, in this regard, is inferior to all those homoeothermic animals that live constantly at high geographic latitudes. The reasons for this are well known: his absence of thick dense wool, a thin layer of subcutaneous fat, and a high metabolic rate with relatively large body size, etc.

Most primates are also characterized by relatively low tolerance to cold stress, with the exception of a small colony of Japanese macaques (*Macaca*

fuscata) that withstand the cold winters of Central Japan. However, their bodies are covered with thick long hair. In contrast, prior to the invention of primitive clothing, housing, and controlled fire, our ancestors lived in places where the ambient temperature did not fall below +10 °C.

Unfortunately, the surface of the Earth, unlike a cartographic map, is uneven. One third of terrestrial land is covered with mountains, sometimes reaching up to 8,000 m and more above sea level. Nevertheless, over the past few millennia man has managed to populate the lower levels of the world's mountains to an altitude of 4,200 m. As it turns out, of all the climatogeographic territories settled by man, the high altitude areas (more than 2,500 m above sea level) have proved the most severe. For endotherms, to which man belongs, high altitudes show: 1) low average annual atmospheric temperatures, which require, among other things, increased energy consumption; 2) low partial pressure of pO2, limiting locomotor activity, and heat production by reducing muscle contractions. Thus, at high altitudes man faces a double danger: he is forced to consume and spend more energy more intensively than in the conditions of lowlands and he has to do all this in conditions of constant oxygen deficiency. In addition, at high altitudes we find dry air, increased radiation, and frequent winds, etc.

In studying the genetic basis of the adaptive evolution of man, scientific approaches sufficiently tested by experience and time has not yet been developed, we are able to focus on the methodology of the general theory of evolutionary genetics. Lewontin [1974] suggests that: "The alternative natural populations to direct measurements of fitness or fitness components is to show at least that selection must be operating, even so it cannot be measured, by correlating the frequencies of alternative alleles with temporal or spatial differences in environment". In other words, in the first approximation, it is possible to rely on both the general similarity and differences in the frequencies of the studied genetic material between populations living in different climatic and geographical conditions. If populations differ significantly in the frequencies of a trait under study, we can postulate local adaptation, but if they are very similar, we can assume general adaptation.

Traditionally, the biology of adaptive human evolution has been studied at the molecular, chromosomal, and organismic levels using the full range of methods of modern medical and biological sciences. This book is devoted to the description of adaptive human evolution at the level of chromosomes and the whole organism.

PART II

THE FACTS

"Patterning of chromosomes is of far greater importance than hitherto realized, yet we have not yet found the tools permitting us to analyze this phenomenon"
—Ernst Mayr [2002].

2.1. Chromosomal heterochromatin regions (HRs) and their variability

Modern human cytogenetics is more than seventy years old. Its biography begins in 1956 when Tjio and Levan first established that the exact number of chromosomes in the human diploid set is 46. The early satisfaction found in establishing the exact number of the diploid set of chromosomes in the human karyotype and the discovery of chromosomal diseases, the so-called "trisomic period" [Lejeune et al. 1959; Ford et al. 1959; Jacobs et al. 1959], soon ran into difficulties once the need arose for accurate identification of each chromosome. The fact is that with the chromosome staining methods of that time, it was only possible to partially identify the human chromosome set. Exactly the same situation was observed in the cytogenetic study of other mammals. This was at a time when insect cytogenetics, using the analysis of polytene chromosomes, achieved impressive success and was already engaged in compiling a physical and genetic map of chromosomes based on the study of their fine morphology. Attempts to use the chronology of DNA synthesis, quantitative measurements of the length of chromosome arms, and other methods for identifying human chromosomes did not give the desired results [Therman 1986].

1968 marked the beginning of a new era in cytogenetics. A group of Swedish scientists led by Caspersson published the results of the first cytochemical studies, indicating that the pattern of differential fluorescence and the length of metaphase chromosomes could be used for full and accurate identification [Caspersson et al. 1968, 1970].

The history of this important methodological discovery certainly deserves a mention. Hsu [1979], the author of the first essay on the history of human and mammalian cytogenetics, and, by the way, one of the authors of the C-staining technique, which initiated the systematic study of heterochromatin regions of eukaryotic chromosomes [Arrighi and Hsu 1971], discussed the origin of the Q-staining technique of chromosomes. At the end of the 1960s, Prof. Torbjörn Caspersson was invited to attend the Children's Cancer Research Foundation in Boston as a consultant. It was unlikely that S. Farber, the director of this Foundation, who invited T. Caspersson, an outstanding cytologist and recognized authority in the field of fluorimetry and interferometry, knew that his choice would have such revolutionary consequences for the development of cytogenetics, particularly human cytogenetics.

By that time it was known that many fluorochromes can stain chromosomes and fluoresce under ultraviolet irradiation. However, all these fluorochromes showed a uniform fluorescence along the entire length of the chromosome. Caspersson, however, focused on whether it was possible to attach reagents to the fluorochrome molecule so that the first could connect to guanines in the chromosomes and the second give fluorescence. Additionally, if the distribution of base pairs along the chromosomes were not accidental, then those parts rich in GC pairs would bind more actively with molecular dyes than chromosomes rich in AT pairs, while detecting bright fluorescence (as well as the fact that knowing the ratio of GC/AT pairs was more important then than now). Therefore, with the validity of all these assumptions, one may expect that chromosomes under a fluorescent microscope will fluoresce differentially, revealing alternating bright and dark zones. As such, it would be possible to distinguish chromosomes by the pattern of their fluorescence. Caspersson persuaded Ed Modest, an organic chemist from the same institution, to synthesize a suitable compound—quinacrine mustard—which was sent for testing to the Karolinska Institute in Stockholm.

The first series of experiments was carried out on plant objects (*Scilla*, *Vicia*, etc.) and under a fluorescent microscope metaphase chromosomes were clearly differentiated by length. At the same time, each chromosome had its own individual alternating pattern of bright and dark, of weak or non-fluorescent segments, which made it possible to completely and accurately identify homologues [Caspersson et al. 1968, 1969a, b].

The first article by the Caspersson group presented pictures of the differential fluorescence of the chromosomes in *Vicia faba*, Chinese hamsters, and *Trillium erectum*, demonstrating the ability to accurately identify each homologous pair in the karyotype. Two years later, this

group adapted a differential staining method to identify the metaphase chromosomes of the human karyotype—the Q-banding (quinacrine-based banding of chromosomes) approach introduced by Lore Zech [Caspersson et al. 1970]. Later, the Paris Conference on Standardization in Human Cytogenetics recommended this technique be named Q-staining (from English "quinacrine mustard") and chromosome segments giving fluorescence were called "Q-bands" [Paris conference 1971].

It is well known that in the use of routine (uniform) color, not all human chromosomes differ sufficiently in total length, nor in the ratio of the lengths of short and long arms, making it difficult to fully identify them. After staining with quinacrine mustard (Q-staining), human chromosomes take on the form of a transversely striated structure in which one can clearly distinguish the segments—from areas that almost non-fluoresce to those with the high (brilliant) intensity. The alternation of such differentially fluorescing segments for each homologous pair of chromosomes is so individual that it makes it possible to accurately identify any metaphase chromosome in the human karyotype. Thus, for the first time, it was possible to undertake an exact and complete identification of each individual chromosome—this was a turning point in the development of human cytogenetics. However, the possibilities of the Q-staining method of chromosomes had not yet been exhausted.

It soon became clear that the constancy of the pattern of differential fluorescence along the length of individual homologous pairs of chromosomes is not absolute. It turned out that, in the human karyotype, one or both homologues of some chromosomes can differ in the presence of particularly brightly fluorescent segments. The aspersson group noted that the distal part of the long arm of the Y chromosome, which is well expressed in the interphase nuclei, also fluoresces most clearly [Zech 1969; Caspersson et al. 1970]. The same authors showed, for the first time, the existence of variable fluorescent segments on the pericentromeric region of autosome 3 and the short arms of chromosomes 13-15. The variability of human metaphase chromosomes was noted by many other researchers on these and on the 4, 21, and 22 autosomal pairs [van der Hagen and Berg 1970; Schnedl 1971; Evans et al. 1971; Cervenka et al. 1971; Lin et al. 1971; Moscetti et al. 1971; Pearson 1972; et al.].

Before proceeding to an analysis of the data obtained by the differential staining of chromosomes, it is necessary to provide some brief information about the history of the problem of the hereditary variability (polymorphism) of chromosomes in human populations.

In the first studies on the morphology of human metaphase chromosomes using routine staining methods, it was noted that certain

regions of some chromosomes varied considerably in their microscopic structure without appreciable influence on the phenotype of the carrier. Such variants were found on the long arms of chromosomes 1, 9, and 16; on the short arms of acrocentric chromosomes; and on the long arm of the Y chromosome. In particular, they manifested themselves in the form of stretched and weakly stained areas, secondary constrictions, in the pericentromeric regions of their long arms on chromosomes 1, 9, and 16. Acrocentric chromosomes varied in the presence of the proximal part of the short arm, in the length of the satellite filaments, and in the size of the satellites. Routine methods of coloring have shown the wide variability of the size of the long arm of the Y chromosome. A peculiar and rare morphological variant has been described for chromosome 17, in which secondary constrictions may appear on a short arm giving the impression of the presence of a satellite region there. Many other very rare variants of chromosomes in the human karyotype have also been described. At the same time, numerous researchers have shown that the chromosomal variants described above are stable in ontogenesis and are inherited. The first information about the existence of racial differences in the frequency of chromosome variants (the size of the long arm of the Y chromosome; the inversion of chromosome 9) were also described before the advent of modern methods of differential chromosome staining.

A detailed analysis of the literature on the routine variants of human chromosomes is not part of our task and they are mainly of historical interest. The fact is that studies of human metaphase chromosomes by methods of differential staining have shown that all chromosomes in the human karyotype are potentially polymorphic—this condition is not the exception, but the rule. The data obtained in recent years indicate an unexpectedly high variability (polymorphism) in human chromosomes and the uniqueness of the karyotype of each individual in human populations. Readers interested in studies on chromosomal polymorphism in humans prior to the introduction of modern methods for the differential staining of chromosomes can find comprehensive information in a number of special and review works [de la Chapelle 1961; de la Chapelle et al. 1963; Ferguson-Smith et al. 1962; Court Brown et al. 1965; Therkelsen et al. 1967; Priest et al. 1970; Lubs, Ruddle 1971; Zakharov 1977; Zakharov et al. 1982; Mikelsaar 1979; Prokofyeva-Belgovskaya 1986].

Any great leap in scientific development implies the emergence of new ideas or research methods. In human cytogenetics, new research methods have usually outpaced new ideas. This occurred with the discovery of the phenomenon of chromosomal polymorphism of heterochromatin regions (HRs) in the human karyotype. Arrighi and Hsu [1971] developed a

fundamentally different technique for the differential staining of chromosomal HRs using Giemsa dye, now known as the C-staining technique. This staining selectively reveals one type of constitutive heterochromatin that exists on all human chromosomes. Therefore, chromosomes stained by the C-technique ("constitutive heterochromatin") are commonly called "C-bands" to emphasize the particular type of heterochromatin detected by this method [Paris Conference 1971]. At present, this technique, with small modifications, has been introduced into cytogenetic practice as one of the main methods for studying the variability of chromosomal HRs in eukaryotes, including humans.

Fluorescent polymorphism of chromosomes, differing from quinacrine polymorphism (Q-staining), reveals Hoechst 33 258 fluorochrome and DAPI (or DIPI) [Schnedl et al. 1977]. The benzimidazole derivative, Hoechst 33 258, which is especially actively fluorescent when interacting with A-T nucleotides, also stains human chromosomes unevenly [Raposa and Natarajan 1974], but the definition of differential fluorescence over the length of a chromosome is much worse. Unlike quinacrine, quinacrine-mustard, or other acridine derivatives, when stained using this technique, heterochromatin near the centromeric regions of chromosomes 1, 9, and 16, the short arm of chromosome 15, and the distal part of the long arm of Y chromosome fluoresce. However, this technique is not widely used in studying the variability of Q-HRs in human chromosomes.

A number of informative methods have been developed that reveal the polymorphism of the nucleolus-organizer regions (NORs) of human acrocentric chromosomes. Those NORs, localized in the satellite threads of all acrocentric chromosomes, can be specifically stained by 'silvering' (Ag-staining) [Goodpasture and Bloom 1975; Howell et al. 1975]. It has now been established that nucleolar organizers undergo Ag-staining if they were active in the previous interphase [Miller et al. 1977]. To date, some very important information has been obtained on the nature of Ag-polymorphic variants of chromosomes, on the nature of their inheritance and tissue differences, as well as on the population frequencies of Ag-polymorphic variants [Mikelsaar et al. 1977; Verma et al. 1981]. In addition to the good reproducibility of the method and its availability for conventional unspecialized cytogenetic laboratories, Ag polymorphism is attractive to researchers because the function of the NORs of acrocentric chromosomes is well known and cistrons for 18S and 28S ribosomal DNA are localized.

Thus, based on the data obtained by the above-mentioned methods of chromosomal differential staining, it turns out that there is much wider chromosomal polymorphism in human populations than was expected

based on data obtained using routine staining before the 1970s. The results of recent studies show that there are many variants of chromosomes in populations that can be detected by various methods (differential staining techniques, *in situ* hybridization of nucleic acids, autoradiography, etc.) and the combination of these variants determines the unique karyotype of each human. Therefore, it is no longer possible to consider the human karyotype as the only morphological standard; it is clear that it is a combination of chromosomal variants, with one or another frequency occurring in populations of normal individuals [Prokofyeva-Belgovskaya 1986; Zakharov et al. 1982; Paris Conference 1971, Suppl. 1975; ISCN 1978; Verma 1988; Bhasin 2005]. Numerous studies have shown that the chromosome regions, identified by these methods as heterochromatic regions, actually possess the fundamental properties characteristic of constitutive heterochromatin.

More than ninety years ago, E. Heitz found that the substance making up the bulk of the nucleus—chromatin—consists of two components: 1) euchromatin, which contains genes, and 2) heterochromatin, which does not carry such genetic material [Heitz 1928]. To imagine the significance of this discovery, it is best to turn to the classic work itself. In 1935, in his review "The Structure of Chromosomes and Genes", based on the achievements of genetics and cytology of the time as well as his own research, Heitz [1935] came to the following conclusions:

> "(1) All chromosomes show a longitudinal differentiation into euchromatin and heterochromatin that relates to the genetic properties of each chromosome. (2) The differentiation is specific for each chromosome and is different in the karyogram of each animal and plant species. … (5) Heterochromatin formation and the degree of chromosomal contraction are genetically determined, and heterochromatin is located at corresponding positions of homologous chromosomes. (6) Chromocenters of interphase nuclei result from equilocal positioning of heterochromatin of different chromosomes. (7) Species can be distinguished by their size and pattern of chromatin distribution. (8) Euchromatin is closely connected to gene activity during interphase; heterochromatin corresponds to genetically inert regions. … (11) Sex chromosomes are frequently subject to heterochromatin formation" (cited in Passarge 1979).

Heitz was also the first to suggest a name for the union of cytology and genetics, then distant to each other as scientific fields: cytological genetics.

The very term "heterochromatin" is a "hybrid" of the terms "heteropicnosis" and "heterochromosomes" used to describe the behavior of sex chromosomes in mitosis in cytology. By proposing the term euchromatin, Heitz was referring to those parts of the chromosomes that

are no longer visible under an optical microscope at the end of the telophase. It is not by chance that Heitz specifically clarified the word "heteropicnosis" as a term denoting the differential behavior of the whole chromosome or its part in the prophase and telophase at a certain stage or throughout the whole development cycle of an individual. The term "heterochromosome" refers to the whole chromosome, while "heterochromatin" refers only to the part of the chromosome that remains heteropicnotic in interphase.

Shortly after Heitz's death, Brown [1966] suggested the terms "constitutive" and "facultative" heterochromatin. Prokofyeva-Belgovskaya wrote that "recently it seems most correct to identify heterochromatin as specific chromosome regions whose DNA is formed by clusters of high and medium-repeating, often satellite, non-transcribed nucleotide sequences. The obligatory property of heterochromatin is the condensed state throughout the entire cell cycle, late replication of the DNA contained in it and positive staining by the C-method" [Prokofyeva-Belgovskaya 1986].

In the last collective monograph devoted to heterochromatin, one of the authors writes:

> "There was a time when it could be argued that heterochromatin differed from euchromatin in its behavior but not in its fundamental structure [Baker and Callan 1950; Dyer 1964; Brown 1966]. That time is now past. Constitutive heterochromatin is indeed composed of DNA sequences with distinctive characteristics. Moreover, there are not two classes of heterochromatin, constitutive and facultative, as is still commonly claimed. The use of the noun *heterochromatin* to describe euchromatin that is facultatively inactivated is both misleading and unnecessary. The facultative heterochromatinization of euchromatin, in principle, has more in common with tissue-specific condensation of euchromatin, though it obviously takes place on a more extended scale, both temporally and spatially, within the individual organism and usually to only one sex" [John 1988].

Time has confirmed the validity of almost all the conclusions of E. Heitz, although he may have been mistaken on a few small things, such as in the use of the characteristic of the formation of chromocenters as an important criterion for determining heterochromatin. We now know that chromocenters are not formed in all cell types and this is true for both constitutive heterochromatin and facultative heterochromatinized euchromatin [John 1988], which also does not form chromocenters. Analyzing the results obtained using a simple cytological method of "boiling" ("Kochmethode"), Heitz managed to achieve an understanding of not only the genetic nature of heterochromatin, but also anticipate one

of the main methods of differential chromosome staining—C-staining. After all, in essence, C-staining [Arrighi and Hsu 1971] is a modification of the Heitz "boiling" technique (the stage of preliminary heat treatment of chromosomes before staining).

2.2. Genetic peculiarities of chromosomal HRs

Brown [1966] proposed distinguishing between two types of heterochromatin: constitutive and facultative. Two homologous chromosomes carrying constitutive heterochromatin—one from the father, the other from the mother—have the same behavior and the same morphological appearance during both embryogenesis and in adults. In the case of facultative heterochromatin (X chromosome), the two homologues behave differently: during development (in early embryogenesis), one of the two homologues becomes partially or completely heterochromatinized, while the second remains euchromatic. This difference persists in the adult body. In facultative heterochromatin, genes are in a state of temporary repression. This is due to the fact that euchromatin regions (ER) located near heterochromatin are not despiralized in the interphase nucleus and remain condensed (heterochromatinized) [Lyon 1961]. As for constitutive heterochromatin, it is recognized that it is usually not transcribed, since the DNA of these HRs has a peculiar molecular composition and they contain mostly moderately or highly repetitive nucleotide sequences.

A classic example of facultative heterochromatin is Barr's body, which is the result of the inactivation of one of the two X chromosomes of a female at an early stage of embryogenesis. It is believed that heterochromatinization is an effective mechanism aimed at ensuring that both sexes have only one genetically active X chromosome per cell [Ohno 1967]. Sex chromosomes are examples of facultative or constitutive heterochromatin, at least in insects and mammals. Thus, the Y chromosome, for most of its length, is formed by constitutive heterochromatin. The same applies to the *w* chromosome in birds.

In many species, the X chromosome makes up about 5 % of the genome—this is the case in humans, dogs, and mice, etc. It is believed that these species retained the original X chromosome, characteristic of a common ancestor [Ohno1967]. Some species of rodent have X chromosome of very large size. Ohno [1967] believes that, in the course of evolution, these large X chromosomes were formed as a result of duplications, triplications, or quadruplications fully reproducing X chromosomes. In this case, in addition to the complete inactivation of one of the X chromosomes in the female, heterochromatinization extends to the remaining

euchromatin region of the male's X chromosome, as well as that of the female. As such, the "universal goal" of this heterochromatinization is the preservation of the genetic equivalent of the original X chromosome in each somatic cell. In other words, the dose of genes associated with the X chromosome, the mechanism of which was explained by Lyon in 1961, is compensated for in the female's cells. However, as Prokofyeva-Belgovskaya [1986] quite reasonably noted, the use of the terms "facultative" and "constitutive" heterochromatin [Brown 1966] is incorrect. She considered it appropriate to remove the term "facultative heterochromatin" and replace it with the term "inactivated euchromatin", which more accurately reflects the nature of the structure and its condition, i.e. in this case the ER undergoes "heterochromatinization". The essence of the phenomenon of heterochromatinization is that a portion of the ER close to the heterochromatin acquires the properties of the latter and goes into a condensed state, repressing the activity of the genes localized there. The process of heterochromatinization is reversible, labile, controlled by the genotype, and affected by developmental conditions; it may not appear in all cells, causing the mosaic development of this trait.

Taylor [1960], using radioautography to study the replication pattern of Chinese hamster chromosomes, described the late replication of the DNA of HRs: in the male a significant part of the long arm of the X and Y chromosomes synthesize DNA at the end of the S phase of the cell cycle. In the female, one of the X chromosomes has the same label as the male X chromosome, while the other only labels late. Taylor found in one species of mammal one of the most characteristic genetic properties of chromosomal HRs—late DNA replication—which had been discovered by Lima-de-Faria [1959] a year earlier in insect sex chromosomes. In *Microtus agrestis*, large blocks of heterochromatin are replicated during the last four hours of the S phase [Pera 1968].

Late replication of heterochromatin DNA is also characteristic of human chromosomal HRs and is a stable property of the X chromosome in women and the Y chromosome in men. In addition, each chromosome in the human karyotype has an area that replicates at the end of the S phase; therefore, these areas see a characteristic localization of the label on radioautographs, allowing us to identify many of them [Schmid 1963; German 1964; Ganner and Evans 1971; Schnedl 1972]. Breg et al. [1972] established that the Q+ and G+ bands mainly correspond to late replicating regions of chromosomes. These observations are consistent with Comings's [1972, 1973] description of the "intercalary" type of heterochromatin. According to the author, intercalary heterochromatin should be distributed along the length of the chromosome arms and correspond to the Q+ and

G+ bands of chromosomes. According to Comings, the DNA of intercalary heterochromatin contains only a small number of highly repetitive nucleotide sequences.

It is believed that there is a correlation between the physical state of chromatin (condensation) and its genetic activity. Regarding the giant chromosomes of Drosophila, it is known that heterochromatin does not take any part in the formation of the characteristic morphological formations in which structural genes are located. Segments of giant (polytene) chromosomes with their characteristic pattern (transverse banding) only form euchromatin regions. As for the HRs of the Drosophila chromosome, which is one-third composed of heterochromatin, it has been established that they do not participate in the formation of characteristic segments where structural genes are localized. It has long been known that the loss of a significant part of chromosomal HRs is usually not lethal, in contrast to the euchromatin material of chromosomes where the loss of even the smallest part is fatal. In addition, the duplication of extensive heterochromatin segments of chromosomes does not cause significant disruption of genetic homeostasis, which also contrasts with the serious consequences of the duplication of ERs in chromosomes.

Finally, crossing-over, the main genetic process of meiosis, usually affects only the euchromatin regions of the chromosomes. Chiasmas, which are the morphological manifestation of this process, are only exceptionally observed in the HRs of chromosomes. The absence of chiasm has been shown in species where chromosomes have extensive heterochromatin zones, for example, hedgehogs and field mice. This has been confirmed at the molecular level [Hotta and Stern 1978]. These authors showed that satellite DNA, which is an important component of heterochromatin, does not include a radioactive label at the pachytene stage. In view of the fact that this stage is characterized by residual DNA synthesis, necessary for recombination, it can be concluded that it does not take place in heterochromatin.

Radioautographic studies of DNA transcription have also revealed the genetic inertness of heterochromatin. Sieger et al. [1970] showed that in the constitutive heterochromatin nuclei of *Microtus agrestis* no radioactive label is detected after the introduction of tritiated uridine, as in the characteristic chromocenter of the nuclei of somatic cells of the quail [Mirre and Stahl 1978].

All these observations suggest that heterochromatin is a genetically inert material in eukaryotic chromosomes. However, the question still arises as to whether this this almost universally accepted hypothesis, in its absolute form, is too great a simplification of the problems arising in the

study of heterochromatin? Indeed, it has long been known that the genetic activity of euchromatin may be altered or violated, and even completely inhibited, if, due to mutation, some part of the heterochromatin is located near euchromatin sites (the "position effect"). It has also been noted that the amount of heterochromatin contained in the nucleus affects the manifestation of some quantitative traits of the organism [Prokofyeva-Belgovskaya 1986; John 1988]. There are other data indicating that chromosomal HRs are far from inert genetic material, the description of which is the primary focus of this work.

2.3. Cytological peculiarities of chromosomal HRs

Currently, the existing methods of differential staining of chromosomes can identify two types of constitutive heterochromatin: C and Q. Several methods are used to identify chromosomal C-heterochromatin regions (C-HRs). The most commonly used method is C-staining [Arrighi and Hsu 1971; Sumner 1972], which makes it possible to stain pericentromeric C-HRs of chromosomes 1, 9, and 16, as well as HRs on the distal part of the long arm of the Y chromosome. The method, called "G-11" [Gagne and Laberge 1972; Bobrow et al. 1972], selectively stains C-heterochromatin in the area of secondary constriction of the long arm of chromosome 9.

The method of Q-staining is mainly used to identify chromosomal Q-HRs, [Caspersson et al. 1970]. DAPI fluorescence (4,6-diaminido-2-phenylindole), through the action of distamycin, reveals HRs in areas of secondary constriction on the long arms of autosomes 1, 9, and 16, the q12 segment of the Y chromosome, and the short arm of chromosome 15 [Miller et al. 1974].

The combination of these methods has allowed us to reach a new level in the study of chromosomal HRs. However, it should be noted that, as Prokofyeva-Belgovskaya [1986] rightly emphasized, "by the 50s our information about the microscopic structure and properties of heterochromatic regions of chromosomes was quite complete. It was obvious that these areas of chromosomes are specialized areas of chromosomes, firmly fixed in them in the process of evolution and clearly distinguished from euchromatic structures by a number of properties". One of the genetic peculiarities of chromosomal HRs has been considered above. Here, we summarize some other cytological features of HRs detected before the 1970s, i.e. before the advent of modern methods of differential staining:

1) All chromosomal HRs are capable of conjugating to each other, forming chromocenters and loops inside the chromosomes and terminal telomeric compounds. This suggests that HRs form if not identical, then biochemically very similar, loci and their conjugation should be considered true homologous conjugation [Prokofyeva-Belgovskaya 1986]. The aggregation of HRs was also observed by Heitz [I934]. He showed that the HRs of the giant Drosophila chromosomes merge to form a bulky chromocenter;
2) HRs under the influence of certain environmental factors (such as cold, illness, and hunger) can be partially despiralized and their DNA concentration decreases. Darlington and La Cour [1940], under cold conditions, were able to localize HRs in the chromosomes of many plants. The euchromatin sites of chromosomes are very resistant to changing environmental conditions;
3) Under the influence of mutagenic factors, as well as some viruses that sever the chromosome, chromosomal HRs are damaged [Natarajan and Ahnström I972];
4) HRs occupy vital areas of chromosomes: centromeres, telomeric ends of chromosomes, regions of nucleolar organizers, and most of the sex chromosomes. With the help of modern techniques of differential staining, it has been possible to confirm that the genetic, cytological, and molecular features of chromosomal heterochromatic material, previously established for different types of eukaryotes, are also characteristic of human chromosomal HRs. The application of these methods has led to the development of a series of new observations of interest.

The most significant of these has been the detection of the wide hereditary variability of chromosomal HRs (see Part II). Data were obtained on the "activity" of heterochromatin in interphase nuclei. In mice, for example, it forms dense chromatin masses located near the nuclear envelope and around the nucleolus [Rae and Francke 1972]. Using radioautography with the inclusion of tritium-labeled thymidine, Comings and Okada [1973] localized heterochromatin by its late label in *Microtus agrestis*, on the one hand, in the chromocenters corresponding to the sex chromosomes, and on the other hand, in peripheral chromatin, which, according to the authors, corresponds to intercalary heterochromatin. The peripheral localization of most of the heterochromatin in the nucleus was also proved for plant cells through the pattern of its late replication in radioautographies with particularly high resolution [Sparvoli et al. 1977].

2.4. Molecular peculiarities of chromosomal HRs

Even before the advent of modern methods of analyzing the longitudinal chemical differentiation of chromosomes (methods of *in situ* hybridization, radioautography, selective fluorescence, and immunochemistry) based on the genetic and cytological features of heterochromatin, the notion that chromosome HRs are formed by a series of numerous, if not identical, biochemically very similar loci with small, complementary effects on some quantitative characteristics of the organism, was developed [Prokofyev-Belgovskaya 1986; Mather I944]. It was postulated that the analogy of the biochemical composition of heterochromatin underlies the attraction, aggregation, and non-homologous pairing of chromosomal HRs.

In this regard, the conclusion of Britten and Kohne [1968] that the DNA of higher organisms contains highly repetitive nucleotide sequences was decisive. At the same time, many cytogeneticists suggested that this class of DNA would be concentrated mainly in chromosomal HRs. The first studies of the biochemical composition of the DNA contained in heterochromatin seemed to confirm the hypothesis of the similarity of HR DNA sequences. Fractionation of DNA isolated from euchromatin and heterochromatin during equilibrium centrifugation on hydroxylapatite, separately and in combination, highlighted the significant heterogeneity of human DNA. Such studies carried out on the cell nuclei of mice and other mammals showed that the DNA of these animals was significantly enriched by satellite DNA—a type of DNA with repetitive nucleotide sequences. Corneo et al. [1967, 1968] found that when DNA was centrifuged on a density gradient of neutral CsCl, two peaks were found corresponding to primary DNA, with a density of 1.700 g/cm3, and satellite I, with a density of 1.687 g/cm3. Yasmineh and Yunis [1973] showed that heterochromatin in mice consists of satellite DNA, and seems to be of the same type, which could, in fact, be the molecular basis of the cytological properties of HRs. However, subsequent studies have shown the improbability of this hypothesis, because it turns out that heterochromatin has a complex and heterogeneous DNA composition.

In 1970, Pardue and Gall, as well as Jones [1970], showed that satellite DNA was localized in centromere regions by *in situ* hybridization on mouse chromosome preparations. At least four types of satellite DNA (satellite I-IV) have been identified in human DNA. Satellite DNA I is present in high concentrations in the distal region of the long arm of the Y chromosome and weakly in the centromeric heterochromatin, less frequently in the telomeric regions of chromosomes 1, 3-5, 9, 12-17, and

19-22 [Jones et al. 1974]. The HRs of chromosomes 1, 3, 13, 14, 16, and 21 were the most constantly labeled. The authors note that this type of satellite DNA is localized in those segments of chromosomes that usually show bright fluorescence after staining with quinacrine.

Satellite DNA II, rich in A-T pairs of nitrogenous bases, is mainly localized in the area of secondary constriction of chromosomes 1 and 16, and, to a lesser extent, in a similar region of chromosome 9 [Jones and Corneo 1971]. A significantly smaller number of satellite II is located in the pericentromeric regions of the chromosomes of groups D, F, and G. The affiliation of satellite II to heterochromatin is proven by the fact that, in the interphase nuclei, RNA complementary to satellite II is localized mainly in chromocenters. Satellite IV is contained in the HRs of many autosomes, especially in the acrocentric and Y chromosomes [Gosden et al. 1975].

Evans et al. [1974] and Gosden et al. [1975] showed that different satellites have preferential localization in different chromosomes. At the same time, Y chromosomes are especially distinguished, containing satellite I and IV, chromosomes 1 and 16 with a predominant localization of satellite II, and chromosome 9 especially rich in satellite III. From the above, we can conclude that satellite DNA is found in all regions of chromosomes formed by constitutive heterochromatin. Miklos and John [1979] noted that in humans, satellite DNA is only 4 % of the genome, while the total amount of constitutive heterochromatin detected by the C-staining method is approximately 20 % of the genome. Thus, it should be recognized that heterochromatin consists not only of satellite DNA. Analysis of human DNA using restriction enzymes revealed the existence of a wide variety of highly repetitive sequences that are not satellites. Manuelidis [1978] was able to show that a DNA fragment of 340 base pairs, isolated by the restriction enzyme *Eco RI*, is concentrated in C-segments of chromosomes, and satellites I-IV (chromosomes 7, 10, and 19) were not found in the heterochromatin of chromosome 1.

New research has suggested that human heterochromatin is heterogeneous in its molecular composition and simultaneously contains satellite and other types of DNA with highly repetitive nucleotide sequences. Studies performed on Y chromosome heterochromatin are promising. They have allowed us to identify the existence of highly repetitive DNA sequences, characteristic only for this chromosome. By reassociating the DNA of a man with an excess of the DNA of a woman, Kunkel et al. [1976, 1977] showed that male DNA sequences, remaining single-stranded, can reassociate with male DNA, but not with female DNA. Experiments conducted with isolated DNA from cells containing the aberrant Y

chromosome, allowed the localization of the "male" DNA sequences on the long arm of this chromosome. Using a different method, Cooke [1976] isolated this type of DNA with restriction enzymes. Using electrophoresis, after the application of the enzyme Hae III, the author identified two segments, being DNA fragments corresponding to sequences 3.4 × 103 and 2.1 × 103, and consisting of approximately 3,000 nucleotide pairs (copies). These segments are not found in DNA of female origin. Through appropriate purification, two specific DNA fractions of the Y chromosome, called Fr Y1 DNA and Fr Y2 DNA, were obtained. McKay et al. [1978], studying individuals who had Y chromosomes of various sizes, showed that these fractions of DNA are localized in long arm heterochromatin and that the amount of DNA corresponds to the length of the heterochromatin segment of the Y chromosome.

Mitchel and Bostock [1977] analyzed satellite III using the restriction enzyme Hae III and found that it consists of monomers 200 nucleotide pairs in length. Satellite DNA III in normal men contains two large fractions that are absent in DNA in normal women. Bostock et al. [I978] showed that the male-specific DNA sequence has a size of 3,500 nucleotide pairs; it repeats about 1,000 times on the Y chromosome and makes up almost I5 % of the mass of the Y chromosome. Experiments with aberrant Y chromosomes have shown that Y-specific DNA is probably localized in the weakly fluorescent region (Y q12) of the long arm of the Y chromosome, which is closely adjacent to the brightly fluorescent region.

The above information regarding the chemical characteristics of the DNA of HRs and their localization in the human karyotype was obtained primarily using the *in situ* hybridization method of nucleic acids [Gall and Pardue 1969; John et al. 1969]. Some additional information was obtained through radioautography, fluorescence, and immunochemistry. The theoretical basis of the first method is the idea that labeled H3-thymidine and H3-deoxycytidine are selectively included in parts of chromosomes whose DNA is rich in either A-T or G-C pairs of nitrogenous bases.

The fluorochrome method, as a tool for analyzing the composition of the nitrogenous bases of DNA in the chromosome, begins with the work of Caspersson and his collaborators [1968] who used quinacrine-mustard in the hope of identifying sites enriched with guanine. Furthermore, this method, based on different degrees of fluorescence depending on the composition of the nitrogenous bases of the DNA, has included the use of acridine [Weisblum and Haseth 1972; Selander 1973], benzimidazole derivatives [Weisblum 1974], and proflavine [Disteche and Bontemps I974].