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A View of an Elemental Naturalist at the DNA World (Base Composition, Sequences, Methylation)

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Abstract—The pioneering data on base composition and pyrimidine sequences in DNA of pro- and eukaryotes are considered, and their significance for the origin of genosystematics is discussed. The modern views on specificity and functional role of enzymatic DNA methylation in eukaryotes are described. DNA methylation controls all genetic functions and is a mechanism of cellular differentiation and gene silencing. A model of regulation of DNA replication by methylation is suggested. Adenine DNA methylation in higher eukaryotes (higher plants) was first observed, and it was established that one and the same gene can be methylated at both cytosine and adenine moieties. Thus, there are at least two different and seemingly interdependent DNA methylation systems present in eukaryotic cells. The first eukaryotic adenine DNA-methyltransferase is isolated from wheat seedlings and described: the enzyme methylates DNA with formation of N^6-methyladenine in the sequence TGATCA → TG**m^{6}A**TCA. It is found that higher plants have endonucleases that are dependent on S-adenosyl-L-methionine (SAM) and sensitive to DNA methylation status. Therefore, as in bacteria, plants seem to have a restriction–modification (R–M) system. A system of conjugated up- and down-regulation of SAM-dependent endonucleases by SAM modulations is found in plants. Revelation of an essential role of DNA methylation in regulation of genetic processes is a fundament of materialization of epigenetics and epigenomics.

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Fifty years ago, the article "A study of species specificity of nucleic acids in bacteria" by A. S. Spirin, A. N. Belozersky, N. V. Shugaeva, and B. F. Vanyushin appeared in *Biokhimiya* [1]. At the first glance of that period, it could be considered as a sort of ordinary regular paper but, in fact, it turned to be an extraordinary one since it contained a few observations very important or even crucial for modern biology. First, it was found that nucleotide composition of total RNA of the cell (bacterial organism) does not correspond to that of total DNA. Thus, this was a true indication for the existence of noncoding RNAs. Second, the comparative analysis of nucleotide compositions of total RNA and DNA of various microorganisms described there, nevertheless, revealed the positive correlation between these nucleic acids on base compositions. This fact showed that some small RNA portion in the cell should fully correspond to DNA according to base composition. Thus, the existence of messenger RNA was predicted. These discoveries were immediately noticed; they significantly influenced the world scientific community, strongly stimulated research in the nucleic acid field, served for development of molecular biology, and were highly appreciated by leading world scientific authorities at the proper time.

Besides, this fundamental work along with other data on bacterial DNA composition appeared practically at the same time from Pasteur Institute in Paris [2], in fact, formed the foundation for genosystematics. It was clearly demonstrated that DNA composition has taxonomic significance and is associated with evolution of bacteria.

My undergraduate student's contribution to this particular research was very moderate—it was limited to investigation of total RNA and DNA compositions of only three bacteria (*Bact. pyocyaneum* (*Pseudomonas aeruginosa*), *Proteus morganii*, and *Proteus vulgaris*). Safe determination of DNA composition at that time was

Abbreviations: m^{6}A) N^6-methyladenine; m^{5}C) 5-methylcytosine; SAH) S-adenosyl-L-homocysteine; SAM) S-adenosyl-L-methionine; SiBA) S-isobutyladenosine.

already reliable due to methods of chromatographic separation and spectrophotometric evaluation of individual bases developed by Dr. E. Chargaff and his coworkers [3]. They were first to detect differences in base compositions between various DNAs (isolated from yeast and avian tuberculosis bacteria) [4]. Based on this observation, Chargaff formulated the conception of DNA species specificity [5]. He considered species specificity of nucleic acids as the difference in DNA base compositions between any two species (organisms) of various origin. But it could not mean at all that differences in DNA base compositions between two species could be obligatorily revealed, and it was not yet clear to what degree the DNA base composition is associated with taxonomy and evolution of organisms. Therefore, it was essential to establish what kind of total DNA, in general, are present or specific for various bacteria, lower and higher plants, animals, and other organisms and what are the limits of variations of their DNA base composition.

Magasanik's method of paper chromatographic separation of monoribonucleotides appeared then [6]; this procedure remains in my memory forever due to the horrible smell of the isobutyric acid that was an obligatory component of the chromatographic solvent. Fortunately, I then developed my own delicate method of ribomononucleotide separation by electrophoresis on paper strips in a special plastic chamber constructed and produced in the mechanical workshop of the Biological Faculty of Moscow State University. This accelerated analysis of RNA composition and made it quite useful and nontoxic; this technique developed by us served efficiently for many years to many scientists in Russia and abroad.

The selection of the first bacteria for my analysis of RNA and DNA nucleotide compositions was quite accidental. Without our own serious microbiological facilities in the Department of Plant Biochemistry, we were glad and thankful to get any bacterial mass from outside. Well-known medical microbiologists Dariya Gavrilovna Koudlay and Adelina Genrichovna Skavronskaya from N. F. Gamaleya Institute of Medical Microbiology and Epidemiology (Russian Academy of Medical Sciences, Moscow) supplied me with blue purulent bacterium (*Ps. aeruginosa*) and proteus. I recall how these charming ladies carefully discussed what kind of bacterial mass they were going to present to Belozersky's undergraduate student. It was all the same for them and for me and finally with one mind they announced: "Let us give him something that is visually most attractive and colorful". As a result, I was proud to bring in ethanol some deep-blue bacterial mass that was used for DNA and RNA base composition analysis. We often used various biological materials presented kindly to us by our colleges and friends mainly from the Biological Faculty of our University (the late Eugene Chinnov and Lev Kuznetsov from the Department of Lower Plants, Dr. G. G. Zharikova from Laboratory of Antibiotics, the late Academician E. N. Kondratieva from the Department of Microbiology, Dr. V. Filin from the Department of Higher Plants; the young chemist A. A. Bogdanov (now Academician) helped us to collect myxomycetes in Moscow region, and so on). This does not mean at all that we did not grow or did not collect especially various biological materials, and we do recall with great pleasure our work associated with collection and primary preparation of different plants including unique ones in Batumi (Georgia) and the Nikita Botanical Gardens (Crimea). Very many different biological objects (including unique bacteria and phages) were especially prepared and supplied to us for mutual DNA research by various USSR and foreign scientists (Yu. M. Azizov, A. V. Alessenko, I. V. Aseeva, N. N. Belyaeva, G. D. Berdyshev, V. P. Bozhenko, S. N. Borkhsenius, L. I. Vorobjeva, A. A. Galoyan, V. N. Gershanovich, D. M. Goldfarb, V. I. Duda, I. I. Nikolskaya, A. A. Kolesnikov, G. F. Koromyslov, L. K. Obukhova, N. P. Palmina, V. B. Rosen, R. I. Salganik, A. S. Tikhonenko, N. A. Tushmalova, N. A. Fedorov, L. A. Chvojka, A. N. Khokhlov, I. Ya. Khudyakov, R. I. Chumakova, N. G. Shuppe, and many others).

DNA base composition as a taxonomic index. Bacteria. The accidental selection of the first bacterial species suggested by D. G. Koudlay and A. G. Skavronskaya for investigation in my student's Diploma work turned out to be especially successful. Even having in mind only three bacterial species mentioned, one can see that in bacteria DNA are strongly different in base composition (GC-content in DNA of *Pseudomonas aeruginosa* is 67% but in vulgar proteus it is 1.7 times lower, 40%) [1]. Also, our data immediately showed that taxonomy of some bacteria can be unfounded and data on DNA base composition may be essential for some further clarifications of position of microorganisms in the system. In the article mentioned [1] we established that DNA of *Proteus morganii* (GC = 53%) very strongly differs from DNA of *Proteus vulgaris* (GC = 40.5%). This allowed us to suggest that *Proteus morganii* seems to be wrongly placed into genus *Proteus*. This corresponded to opinion of some microbiologists, who suggested that *P. morganii* should be transferred into genus *Morganella*. Soon it was also suggested that bacterial species with differences in DNA base composition more than 10% GC cannot be united in one and the same genus [7]. We established that parasitic and saprophyte forms of *Bdellovibrio bacteriovorus* are different in DNA base composition [8], and significant differences in DNA base composition were found between DNA from vegetative cells and spores in anaerobic bacteria [9].

The massive consecutive systematic study of various DNAs strongly supported the idea that DNA base composition is an important taxonomic feature, and the world-famous "Bergey's Manuals of Bacteriology"

stopped registering new bacterial species without data on their DNA GC-content. Therefore, many Soviet microbiologists addressed us asking for determination of DNA base composition in different original microbes isolated first by them. Besides, DNA base composition in representatives of many whole large taxonomical groups of bacteria was still unknown. Thus, we were first to determine DNA base composition in many chemosynthetic [10], photosynthetic [11], luminescent photobacteria [12], propionic bacteria [13], cocci-form microbes isolated from lithophilous lichens [14], and others. The comparative investigation of nucleic acids in all these microorganisms was important for clarification of their taxonomy as well as of origin and evolution of different functions—especially such as photosynthesis, chemosynthesis, and fixation of molecular nitrogen. Similarly to heterotrophic bacteria, here were many curious observations. For example, the differences in DNA base compositions between *Nitrosomonas* species and pseudomonades [10] indicated that the genus *Nitrosomonas* should be considered as a family Nitrosomonadaceae. The sulfur and non-sulfur photosynthetic bacteria turned out to be very similar in DNA base composition; the most likely origin and evolutionary relations between purple, green photosynthetic bacteria, thiobacteria [11], and *Azotobacter* [10] were suggested. According to DNA base composition, the luminescent photobacteria of genus *Photobacterium* we divided into at least two groups, and one of the new species was named as *Photobacterium belozerskii* [12].

Surprises from phage DNAs. Despite the fact that the nature and composition of phage DNAs were investigated in only very few bacteriophages (when we started our own work with them), nevertheless, it was already known that these peculiar DNAs may fundamentally differ in the base nature from DNA of their bacterial hosts. So, in T-even bacteriophages cytosine is substituted by 5-hydroxymethylcytosine that sometime in addition may be glucosylated. In some phage DNAs, thymine is substituted by hydroxymethyluracyl. In DNA of XP12 phage grown on *Xanthomonas oryzae* cells all cytosine is substituted by 5-methylcytosine [15]. We have been interested in such "fully methylated" DNA and asked our colleagues from Taiwan to send us this phage and the host. Unfortunately, the parcel received was broken and we could not recover these interesting biological materials. When we analyzed DNA structure of AR9 bacteriophage grown on *Bac. subtilis*, thymine was not found in this DNA because it was fully substituted by uracil [16]. Thus, since that time uracil is not to be considered as a unique feature of RNA because it may be a real ordinary base in some DNAs. This uracil-containing phage DNA melted at much lower temperature compared with respective equivalent in GC-content normal thymine-containing DNA. In principle, it was the first reliable indication that methyl groups of pyrimidine bases in DNA stabilize its secondary structure. Double-stranded cyanophage S-2 DNA does not have adenine; instead it was represented by 2,6-diaminopurine (2-aminoadenine) [17]. This base unusual for natural DNAs imparts some unique properties to phage DNA: this DNA melts at higher temperature compared with normal DNA of the same GC-content (in particular, this is due to the fact that all base pairs have three hydrogen bonds in such DNA); it is resistant to many known restriction endonucleases that split DNAs with ordinary bases.

Eukaryotes. As already mentioned, the data on DNA base composition of eukaryotes were very scanty when we started our DNA research. DNA base composition in representatives of the whole Type of myxomycetes (slime molds) was completely unknown. DNA of the gigantic world of fungi was limited to only yeast DNA. Higher plant DNA base composition was represented only by data on wheat germ DNA. Our task was to answer the question, at least in part, what kind of DNA do these eukaryotes have?

The first comparative and more or less comprehensive data on the nature of DNA of fungi and myxomycetes were obtained in Russia [18, 19]. Similar to bacteria but to lesser degree, the DNA base composition (GC-content) in fungi strongly varied (at least twofold) between various species. Significant differences in DNA GC-content were observed in lower fungi (phycomycetes), ascomycetes, fungi imperfecti, and basidiomycetes. Very often they were detected even in species of one and the same genus. Even at that period, we suggested that data on DNA base composition may be useful for identification of some fungi imperfecti. These early suggestions were appreciated by many mycologists.

The first systematic studies of DNA base composition in higher plants were also performed in Russia. These data concerned DNA of archegoniates (mosses, horsetails, ferns, gymnosperms) and flowering plants (mono- and dicots) [20, 21]. Variability of DNA base composition in higher plants was much less compared with that of bacteria and fungi. Nevertheless, in mosses and other plants, species differences in GC and 5-methylcytosine contents were detected. Some plants with total DNA of GC-type were first found among mosses.

Early investigations of DNA base composition induced and directed by A. N. Belozersky strongly extended the world's knowledge of the DNA nature in invertebrates (sponges, mollusks, coelenterates, arthropoda, echinodermata, and others) and vertebrates (cyclostomes, fish, amphibia, reptiles, birds, mammals) from different taxonomic groups [22-24]. It became clear that DNA base composition is a conservative characteristic that reflects to some degree a hereditary specificity of organisms, and variability in limits of DNA base composition are directly proportional to the evolutionary age of taxons [23]. The main merit of these pioneering works was that they in fact drew attention of different biologists to genomes by real demonstrations showing clearly that

deciphering of DNA structure may be a new elegant and powerful key to solution of global problems of taxonomy and the origin and evolution of organisms—it is the real foundation of genosystematics.

First steps in the analysis of the nucleotide sequence of DNA. While data on DNA base composition generated genosystematics, they were indicative only and insufficient for progress of this important new discipline. There was very strong need for determination of DNA primary structure (nucleotide sequence). But there were no methods for DNA sequencing. It was only known that DNA can be hydrolyzed with acid into pyrimidine sequences (fragments) [25], but they should be subsequently fractionated somehow in order to determine their frequencies in genomes of different origin. We developed method of separation of pyrimidine fragments (oligonucleotides) according to their length and base composition by means of DEAE-Sephadex column chromatography [26]. This procedure allowed us to isolate from various DNA and analyze at least eight pyrimidine isopliths (fragments with the same number of nucleotide residues). In parallel, we developed a method of DNA degradation into purine sequences; this was done by DNA hydrazinolysis (removal of pyrimidines) with subsequent treatment of apyrimidinic DNA with aniline [27, 28]. A similar procedure (hydrazinolysis) was in fact a precursor of the DNA sequencing method developed by Gilbert and Maxam. Even before the appearance of real methods for DNA sequencing, we had a unique possibility to compare DNA primary structures by determining in them frequencies of various pyrimidine and purine blocks (clusters), different in length and base composition. In particular, in this way the similarity of N^6-methyladenine-containing sequences was first established in DNA of T_2 phage and its host *E. coli* B [29]. Thus, it was one of the earliest chemical supports for the phenomenon of host DNA modifications in microorganisms. Good analytical correspondence of the data on frequencies of the complementary purine and pyrimidine clusters isolated from one and the same DNA shown that methods of DNA degradation to pyrimidine and purine fragments developed by us were adequate, specific, and they could serve as procedures for analysis of respective (limited) sequences in different DNAs. Analysis of DNAs by these particular methods showed that the distribution pattern of pyrimidines (purines) in different DNA is far from random, and it can be quite different even in DNAs that are even identical in base composition [30, 31]. In fact, these data were the first reasonable attempts to judge to some degree the primary structures of DNA of different origin. By these methods DNA from various bacteria, bacteriophages, fungi, higher plants, and animals (sponges, mollusks, crustacea, echinodermata, holoturia, fish, amphibia, reptiles, birds, and mammals) were investigated. For example, by this analysis we were able to differentiate between DNA from two fungi (*Verticillium* sp., pathogenic and saprophyte non-pathogenic) similar in GC-content [32] and distinguish between total DNA from healthy and wilt-infected cotton plants [33]. We observed that evolution in animals is accompanied by increase in the DNA pyrimidine clustering degree in the series invertebrates–fish–amphibia–reptiles–birds–mammals. It is clear that today these data have mostly historical interest, but they turned out to be important for understanding of general principles of structural organization of genomes. It seems that these first steps toward DNA sequencing were a sort of whole epoch in the investigation of nucleic acids and a necessary stage in the origin and perfection of modern great-scale sequencing of genomes.

Minor bases and DNA methylation. It is well known that DNA may contain some additional minor bases—5-methylcytosine (m^5C) [34, 35] and N^6-methyladenine (m^6A) [36]. These particular bases (m^5C and m^6A) appear in DNA as a result of enzymatic methylation of residues of the respective usual bases. For a long period, the specificity and functional significance of enzymatic DNA methylation were unknown. Moreover, the idea that minor bases do not play any essential role in DNA structure as well as in its functioning was quite common. Erroneous data on the absence of DNA methylation in *Drosophila* due to insensitive methods were used as an "irresistible" argument for such a conception. 5-Methylcytosine in the genome of this insect escaped detection for a very long time. Therefore, there was a false occasion for many investigators to conclude that DNA methylation is not important in the life of eukaryotes as far as *Drosophila* can exist without it. A long time ago we noticed that the *Drosophila* genome is characterized with a strong deficiency in CpG sequences that are the main targets for DNA methylation in eukaryotes; as to our opinion, the strong CpG suppression in the *Drosophila* genome could be due only to methylation of cytosine residues in DNA [37]. As we were not able to detect DNA-methyltransferase activity in *Drosophila* cells at that time [38], we designed such possible DNA modification in an insect as a "fossil" DNA methylation [37]. Now it has been shown that *Drosophila* DNA is methylated and this genome modification is important for insect development and is clearly detected in the early stages of development [39, 40].

We have always been sure that minor bases in DNA and the enzymatic methylation of the genome should influence genome structure and its functioning. It turned out that DNA methylation stabilizes secondary DNA structure as well as having a strong influence on binding of DNA with different proteins—including regulatory ones. Very often DNA methylation inhibits the binding with specific nuclear proteins (factors) that take part in transcription, replication, and DNA repair. Proteins binding specifically with regulatory elements of rRNA genes (135 bp subrepeat element) were found in plant nuclei, the *in vitro* cytosine methylation in CCGG sites of

these elements completely abolishing their ability to bind some of these proteins [41]. On the other hand, there are m^5C-DNA-binding proteins that associate with methylated DNA only, and their binding arranges on DNA a whole ensemble of complicated protein complexes that control and carry out gene expression [42, 43].

When DNA was incubated in the presence of methyl-labeled S-adenosyl-L-methionine (SAM) without any proteins added, radioactivity was detected in DNA and, particularly, in 5-methylcytosine and thymine residues newly formed there. In this way we discovered non-enzymatic DNA methylation [44]. Interestingly, the amount of labeled thymine formed was much more than that of labeled m^5C. Thus, it was shown that non-enzymatic DNA methylation in aqueous solution is conjugated with rapid oxidative deamination of the m^5C residues formed with their transformation into thymine residues. This was real evidence that methylation of cytosine residues in DNA may often result in C → T transition, and 5-methylcytosine residues in DNA are "hot" mutation points. This event is a reason for disappearance (suppression) of some CpG sequences from genes and genomes of various organisms, and it is a main route of natural mutagenesis and evolution.

The existence of potential non-enzymatic DNA methylation in nature was effectively used by specific enzymatic proteins that appeared with evolution; these proteins, DNA-methyltransferases, unlike random non-enzymatic methylation, modify cytosine and adenine residues specifically in definite DNA nucleotide sequences. We deciphered one of the first such sequences methylated in bacterial DNA [45]. It appeared that in *Bacillus brevis* cells the cytosine DNA-methyltransferase methylates a cytosine residue (underlined) in the symmetrical nucleotide sequence (5′) ...N′G<u>C</u>TGCN... (3′). We found that DNA methylation in bacteria is species and strain specific [46]. The DNA methylation character changes on bacterial dissociation (R, S-forms) [46], spore formation [47], and pyocine induction [48]. It seems that these data were first to show that DNA methylation is associated with cell differentiation in microorganisms.

Even before the methods of DNA sequencing appeared we were able to show that in plant genomes 5-methylcytosine is located in sequences such as Pu-m^5C-Pu, Pu-m^5C-T-Pu, Pu-m^5C-C-Pu, and Pu-m^5C-m^5C-Pu [49]. This corresponded to data of Dr. Razin's group that appeared simultaneously on methylation of cytosine residues in plant and animal DNAs in CG- and CNG-sites [50]. According to our data for plant DNA, up to about 30% of 5-methylcytosine is localized in m^5CNG sequences [49]. These sites in particular appear to be methylated during induced small dsRNA-directed methylation of genes that results in their inactivation (gene silencing) [51]. DNA methylation in plants is much richer and more diverse compared with animals; it is carried out by a larger set of specific DNA-methyltransferases, some of which are controlled and utilized via the ubiquitin pathway [52, 53].

As for biological specificity of DNA methylation, we learned a long time ago that it is species-specific. In many invertebrates methylation degree of the genome is very low, whereas in vertebrate DNAs m^5C is always present in noticeable amounts [54]; in plant DNA this base cannot even be called a minor one because it is quite comparable quantitatively with cytosine [21, 55].

We have established that along with species specificity there are also tissue (cellular)-, subcellular (organelle)-, and age-specificity of DNA methylation in animals and plants. It seems to be appropriate to cite here one of our American colleagues, Dr. Craig Cooney [56]: "...The Russians had shown that as animals age, DNA methylation declines. This was a tantalizing suggestion: that loss of DNA methylation and aging went hand in hand... Does this mean that there is a link between cellular aging and decreases in overall DNA methylation? Apparently so. ...Dr. Boris Vanyushin and co-workers in Moscow first showed in the 1960s and published in 1967 that salmon lost DNA methylation with age [57]. The same group of scientists later showed that what was true in salmon was also true for most of the organs they studied in cattle [58-61] and rats [62-65]. Later studies by several groups of scientists in the United States and Japan indicated that many organs in mice also showed loss of DNA methylation with aging" (*references in this citation are included by B. F. V.*). Now the age-dependent demethylation of DNA is quite obvious, and some investigators are inclined even to consider DNA methylation degree as a sort of biological clock that measures age and forecasts lifespan. Distortion in DNA methylation leads to premature aging.

We have observed that DNAs in the cells of one and the same organism are methylated in a different fashion. Detection of tissue specificity of DNA methylation allowed us first to declare that DNA methylation should be a mechanism of regulation of gene expression and cell differentiation [54, 65]. These data have drawn the attention of many investigators in this country and abroad and pushed ahead the intense study of DNA methylation throughout the world.

We also found that DNA methylation in mitochondria and the nucleus of one and the same cell is different [66-68]. Cytosine DNA-methyltransferase from bovine heart mitochondria has different site-specific action compared to the nuclear one [69]. Thus, the subcellular (organelle) specificity of DNA methylation was discovered.

Functional role of DNA methylation. A large body of data on the specificity and biological role of DNA methylation in different organisms is presented in our recent reviews [70-73]. Here we shall remind very briefly only a few of our most important findings. For example, it was observed that DNA methylation pattern in liver changes strongly after hydrocortisone administration into animal,

and this is associated with induction of various genes [61, 64, 74, 75]. DNA methylation pattern in neurons but not other brain cells changed during rat training [76-78]. Changes in the neuronal DNA methylation pattern induced by training were among the first evidences that the genome takes part in memory formation [76]. In plants DNA methylation strongly changes on seed germination [79], transition to flowering [80], and after infection with various fungi, parasitic plants, and viruses [33]. It became clear that infective agents can more or less delicately act on plants in their own favor by modulation of DNA methylation in host cells. "As early as 1977 a Russian team looked at normal cells from cows, then compared those with cells from cows who had a type of cancer known as lympholeukemia. In general, the overall DNA methylation was lower in cells from animals with the cancer. This was one of the early clues that methylation, DNA methylation at least, might be involved in cancer, either as a cause or as a result" [56]. In fact, we have shown that in lymphocytes of cattle suffering from chronic lympholeucosis the DNA methylation pattern is drastically changed compared with normal cells [81-86]. In malignant cells with very high DNA-methyltransferase activity the level of total DNA methylation was lower but in palindromic highly-reiterated sequences it was higher than that in normal lymphocytes [85]. Unlike in normal cells, at least two DNA-methyltransferase activities were found in the nuclei from cattle suffering from lympholeucosis. One of these enzymes differed strongly in site specificity from DNA-methyltransferase activity of lymphocytes from healthy animals [86]. All these findings allowed us to first firmly conclude that serious distortions in DNA methylation are a route to cancer. Now this idea is an axiom; it was confirmed lately and strongly developed now in brilliant works done by Drs. S. Baylin, M. Ehrlich, R. Jaenisch, P. Jones, M. Szyf, G. Pfeifer, W. Doerfler, P. Volpe, and many others. Now the data on distorted DNA methylation pattern are safe diagnostic and early characteristic of some cancer forms. We have observed recently an increased level of *DMT1* gene expression, changes in the set and site specificity of DNA-methyltransferases, and appearance of new proteins with DNA-methyltransferase activities in tumor (hepatoma) cells of rats fed with methyl-deficient diet [87, 88]. Thus, our original idea [81] of the triggering of malignancy by distortion of DNA methylation has been confirmed again.

We have established that DNA methylation in animals is under hormonal (hydrocortisone) control *in vivo* and *in vitro* [61, 64, 69, 74, 75], this genome modification being modulated by antioxidants [64, 87, 88]. On the other hand, the *in vitro* methylation of rat liver DNA with homologous DNA-methyltransferase inhibits DNA binding with hormone–receptor complexes (HRC) [89]. This is a very important observation. It means that some mutual control exists in cells: hormones control DNA methylation but DNA methylation is essential for realization of hormonal signal. This is true both for animals and plants. In plants, DNA methylation is regulated by different plant hormones (phytohormones, specific plant growth regulators) [90-92]. Some phytohormones strongly suppress global DNA methylation in plants. Besides, phytohormones inhibit methylation of newly formed DNA but not the Okazaki fragments [90-93]. This was the first evidence that phytohormones can influence genome by modulation of its methylation status. In general, we are inclined to think that modulation of DNA methylation is one of the principal mechanisms of hormone action in plants and animals. It cannot be ruled out that HRC in plants and animals can recognize and compete well with respective DNA-methyltransferases for specific DNA binding sites.

Discovery of replicative DNA methylation. We have observed that at high cell concentrations in a medium the DNA synthesis in animal and plant cells is mainly limited to formation of the earliest replication structures synthesized—short DNA replication fragments (Okazaki fragments) [94, 95]. It turned out that Okazaki fragments in plants and animals are already methylated [90, 92-95]. Thus, the proper replicative DNA methylation in eukaryotes was discovered and well documented; also, it was suggested that some DNA-methyltransferases can be constituents of the replicative complex [95]. We concluded that the nucleus should contain a few DNA-methyltransferases, each of them serving at a particular replication stage and differing from others in site specificity and sensitivity to respective inhibitors [90, 95]. This is now in full agreement with modern data on the multiplicity of DNA-methyltransferases in plant and animal cell. Then we were able to discriminate between replicative and postreplicative DNA methylations in plants [90, 96-100]. These types of DNA methylation are different in site specificity and regulation by phytohormones and inhibitors.

We have suggested the mechanism of the natural regulation of replication by DNA methylation. Replication of hemimethylated DNA in the cell seems to be prohibited as it can result in the loss of the epigenetic signal [92, 93]. It was much later shown that this regulation of replication is realized in bacteria by *dam* methylation. Now it is more or less clear how DNA methylation pattern can be inherited. As already mentioned, replication produces hemimethylated sites in DNA formed, and most genes seem to be functional when DNA in an interphase nucleus is in this particular state. Before the next round of replication and cell division the maintenance DNA-methyltransferases, particularly dmt1 responsible for maintenance of genome methylation status, methylate hemimethylated sites with formation of fully methylated ones. The m^5C in one DNA chain serves as a signal for methylation of the opposite cytosine residue in a complementary chain. This stops gene transcription but permits

replication when the cell cycle is completed. Thus, DNA methylation pattern is inherited. For instance, treatment of wheat plants with 5-azacytidine (a DNA methylation inhibitor) results in an increase in the seed storage protein content by more than 30% [101]. This seed property is inherited for few plant generations.

Discovery of adenine DNA methylation in higher eukaryotes (higher plants). N^6-Methyladenine has been found in total DNA in many higher plants [102, 103], and, in particular, in wheat mitochondrial DNA [104]; it was shown that, unlike in animals, the mitochondria in plants (wheat) possess adenine DNA-methyltransferases but not cytosine DNA-methyltransferases [105]. In plants a new class of heterogeneous in contour length minicircular heavy mitochondrial DNAs containing N^6-methyladenine but not 5-methylcytosine was observed [106]. These DNAs were found in all archegoniate and flowering plants investigated [107]; synthesis of these DNAs is most strongly pronounced in aging plant organs [106]. Intense replication of these mtDNAs is one of the specific apoptotic features in plants [108, 109]. ORFs homologous to bacterial adenine DNA-methyltransferases are found in nuclei of protozoa, yeasts, insects, nematodes, higher plants, vertebrates, and other eukaryotes [110]. Therefore, adenine DNA methylation may be quite common for eukaryotes. Up to now only one of these eukaryotic enzymes that modifies DNA on adenine residues is known [111]. It has been isolated from wheat cytoplasmic vesicles specific for apoptotic plant cells [109]. The enzyme (wadmtase) methylates an internal adenine residue in the TGATCA sequence predominantly in a single-stranded DNA and seems to take part in regulation of mitochondrial DNA replication [109]. As far as one and the same gene in plants can be methylated at both adenine and cytosine residues [112], it was suggested that there is a system of mutual control for (between) these different genome modifications. Anyway, methylation of adenine residues influences cytosine methylation in plant DNA and *vice versa*. For example, adenine methylation in GATC sites of *DRM2* gene in *Arabidopsis* plants is strongly diminished in the presence of induced antisense constructions of *METI* transgene for cytosine DNA-methyltransferase [112].

The control for adenine DNA methylation by cytokinins (N^6-derivatives of adenine) as a result of their incorporation into DNA may be a mechanism of regulation of gene expression and cellular differentiation in plants [93]. Phytohormones cytokinins (natural adenine derivatives) can be directly incorporated into DNA in plants [113] and the protist *Tetrahymena pyriformis* [114]. Natural incorporation of cytokinins substituting for adenine residues into sites recognized by adenine DNA-methyltransferases should prohibit adenine methylation in these particular sites. Thus, adenine undermethylated DNA may occur and this may result in a significant change in gene transcription and DNA replication.

SAM-dependent plant endonucleases sensitive to DNA methylation status. Being engaged in an intense investigation of nucleic acids for a long period, we unfortunately quite recently realized in practice that research in DNA is far from enough to understand proper gene functioning. Recently we have isolated and partially characterized new Ca^{2+}/Mg^{2+}- and SAM-dependent endonuclease WEN1 with molecular mass about 27 kD from wheat coleoptiles [115]. WEN1 splits double-stranded DNA of phage λ (dam^+, dcm^+) with methylated cytosine residues (in sequence Cm^5CWGG) and methylated adenine residues (Gm^6ATC) more efficiently compared with the same but unmethylated DNA of λ phage (dam^-, dcm^-). In other words, WEN1 distinguishes between methylated and unmethylated DNA—it recognizes methylation status of substrate DNA. This was unknown for endonucleases from higher eukaryotes but is quite specific for bacterial restriction endonucleases. This means that in plants like in bacteria a restriction–modification system could be present. S-Adenosyl-L-methionine (SAM), a universal donor of methyl groups, activates hydrolysis of unmethylated but not methylated λ phage DNA with enzyme WEN1. The competitive inhibitors of DNA methylation, S-adenosyl-L-homocysteine (SAH) and S-isobutyladenosine (SiBA) (unlike SAM they do not have active methyl group) also stimulate this enzymatic hydrolysis of unmethylated DNA. Therefore, the SAM activation of DNA hydrolysis is not associated with methyl-donor properties of SAM—rather S-adenosyl group of SAM, SAH, and SiBA is an allosteric effector of WEN1 endonuclease activity. Thus, it was first shown that plant endonuclease like typical bacterial restriction endonucleases is modulated by SAM and its analogs. Therefore, a new type of regulation of eukaryotic endonucleases by modulation with SAM was discovered. It is not ruled out that WEN1 takes part in degradation of nuclear DNA on apoptosis. Due to many properties (ionic dependence, double pH optimum, and others), plant WEN1 enzyme is reminiscent of animal mitochondrial endonuclease G that takes part in the nuclear DNA fragmentation on apoptosis. Another unique endonuclease isolated very recently from wheat coleoptiles is also a SAM-dependent enzyme, but unlike WEN1, SAM does not activate but inhibits this new enzyme. This is first case with eukaryotic endonucleases when SAM inhibits their activities. Thus, under the conditions favored by SAM for DNA methylations the activity of one endonuclease is inhibited while activity of other enzyme (WEN1) is stimulated. Thus, in plants a system of conjugated regulation of endonucleases by polarity different SAM action and, in particular, of ones sensitive to DNA methylation status was discovered.

DNA methylation and other epigenetic signals. The origin of a new science, epigenetics, is mainly due to observation and description of the exclusive role of DNA methylation in the life of various organisms. The way to

decipher the nature and specificity and to understand the biological role of this enzymatic DNA modification (methylation) was very uneasy and we are glad to realize that our small landmarks on this hard way are also visible. I would like to stress that this research was started under the late Academician A. N. Belozersky and it is always associated with his name.

There are many other different epigenetic signals in a cell. In particular, today we can already speak of "histone code" [116]. DNA methylation and histone modifications are closely connected. In *Neurospora*, the methylation of lysine 9 in H3 histone is critical for cytosine DNA methylation and normal development of the fungus [117]. In other words, histones can be the signal transmitters for genome methylation. On the other hand, in *Arabidopsis* plants CpG DNA methylation precedes and directs lysine 9 methylation in H3 histone [118]. In plants and animals, DNA methylation is associated with histone deacetylation [119, 120]. Indeed, "methylation meets acetylation" [121]. For instance, the histone deacetylase gene is essential for small RNA (dsRNA)-directed DNA methylation. Histone modification induces DNA methylation in CNG sites, and this methylation may be particularly responsible for gene silencing by small RNAs [122].

In addition to many histone modifications modulating chromatin organization and DNA availability to enzymes, many other proteins can effectively compete with DNA-methyltransferases for DNA binding. In particular, various hormone–receptor complexes seem to perform this business. This could explain, at least in part, the mechanism of regulation of DNA methylation by hormones observed in plants and animals. Anyway, further progress in the study of genome methylation in eukaryotes depends now mainly on research in the detail of fine chromatin structure and its various functional modulations in the nucleus.

Thus, our view at the DNA world made initially half a century ago was not just a meditative and fruitless one, it essentially extended our knowledge of the nature and biodiversity of genetic material in general, allowed us to discover some unique features of DNA structures and functioning, laid the foundation of genosystematics, and practically materialized a new science—epigenetics.

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