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Chemical approaches to the study of supramolecular biological structures with chromatin as an example

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Abstract. Chemical approaches to the study of the chromatin structure and certain aspects of its functioning are reviewed. The main attention is given primarily to those based on the method of 'zero-length cross-linking' aimed at the investigation of the structure of histone complexes within nucleosomes and of the interactions of histones and other proteins with chromosomal DNA. The changes in the nucleosome structure during chromatin transcription are discussed. Recent data on the chemical modification of DNA and several chromatin proteins with reactive oligonucleotide derivatives are considered and analysed. The bibliography includes 89 references.

I. Introduction

Chromatin is one of the most complex supramolecular structures of eukaryotic cells which is responsible for the storage, multiplication, repair and expression of genetic information.[1] Chromatin comprises a great number of constituents and its structure manifests high dynamism associated with dramatic changes both during the cell cycle and as cell responses to incoming (mitogenic, *etc*.) signals. The eukaryotic nucleus is made up of several molecules of double-stranded DNA distributed between individual chromosomes. The human haploid genome consists of 3×10^9 base pairs (b.p.). Assuming that this DNA exists entirely in the *B* form where one loop (10 b.p.) has the length of 3.4 nm, the total length of DNA is ~ 1 m, *i.e*., the whole set of 23 chromosome pairs measures ~ 2 m in length. This DNA is packed into a nucleus with a diameter ~ 20 μm. Thus, the degree of compactisation must be of about five orders. In the course of its functioning (replication, transcription, repair, recombination and formation of mitotic chromosomes), DNA is subject to substantial topological rearrangements, so that the array of proteins interacting with it changes drastically.

At the molecular level, the structure and function of chromatin cannot be adequately investigated by physical methods alone. Not only the whole chromatin, even its domains are too large to allow their crystallisation for X-ray analysis, NMR studies or calculation of the DNA structure by the molecular dynamics method. Therefore, chemical methods appear to be highly suitable for the study of chromatin structure. Affinity modification of chromatin seems to hold especially great promise in gaining information about the structural rearrangements occurring in the nucleus during its functioning. In the first place, this refers to photomodification, which allows specific modifications of definite sites of chromatin over a very short time. The latter circumstance opens up new possibilites for the investigation of events that take place during the functioning of chromatin components.

Chromatin represents a nucleoprotein complex of DNA with nuclear proteins, *viz*., with basic proteins (histones) and nonhistone proteins including the most well-studied HMG proteins which are considered in this review. It should be noted that during its functioning the nucleoprotein complex interacts with a large set of proteins involved in matrix synthesis, with nuclear matrix proteins (the structure of the nucleus which remains intact after its dissociation from the central nucleoprotein), *etc*. These compounds may be regarded as chromatin-associated proteins.[1]

The first level of chromatin organisation is the nucleosomal fibril with a diameter of ~ 10 nm. It represents a DNA chain wound with definite periodicity around a complex made up of four histone pairs (an octamer) designated as H2A, H2B, H3 and H4. These discrete repeat units are obtained by mild treatment of nuclei or isolated chromatin with micrococcal nuclease. A complete nucleosome contains DNA of up to 200 b.p. in length. The core particle contains DNA of 145 – 146 b.p. in length, which forms 1.75 turns of the left superhelix around the histone octamer. The rest of the nucleosome is regarded as a linker. The structure of the core nucleosome has been solved by X-ray analysis with a 7 Å resolution.[2] The linker region of DNA of a variable length is complexed with histone H1. This histone seems to play an important role in the formation of the next level of the structural organisation of chromatin, *viz*., the packing of the nucleosomal chain into a solenoid (a fibril which contains six nucleosomes per turn and has a diameter of 30 nm). In addition to histone H1, chromatin of avian, reptilian, amphibian and fish erythrocytes also contains linker histone H5. After removal of all nuclear histones with a concentrated sodium chloride solution containing detergents, DNA remains bound with the nuclear matrix and forms supercoiled loops.[3] It was concluded from these data that DNA is attached to the protein backbone of the nucleus (the nuclear matrix) at definite sites. Electron microscopic studies revealed that after treatment of metaphase chromosomes with 2 M NaCl or an ionic detergent DNA forms loop-shaped domains fixed on the proteins of the chromosome backbone ('scaffold').[4]

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Thus, a fibril with a diameter of 30 nm forms domains appearing as 18 radial loops (each of them has an average length of 60 000 b.p.) that are fixed on the nuclear matrix. These densely stacked rosettes form the next organisational level, *viz*., the chromatid (Fig. 1).[5]

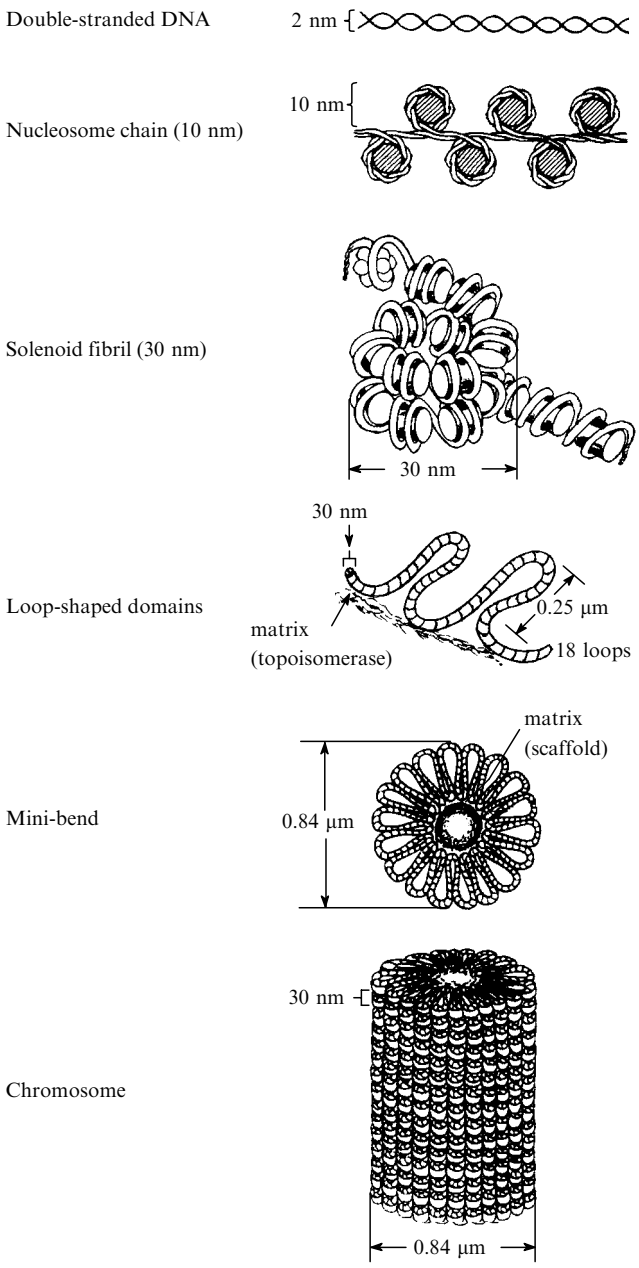


Figure 1. Various levels of chromatin organisation.[5]

The chemical approaches to the study of nucleosome structure were used to solve three main problems, *viz*., to establish the structure of histone complexes, to identify the sites of DNA interaction with histones and to study the rearrangements in chromatin resulting from the transition of genes into an active or inactive state.

II. Study of interhistone interactions in chromatin

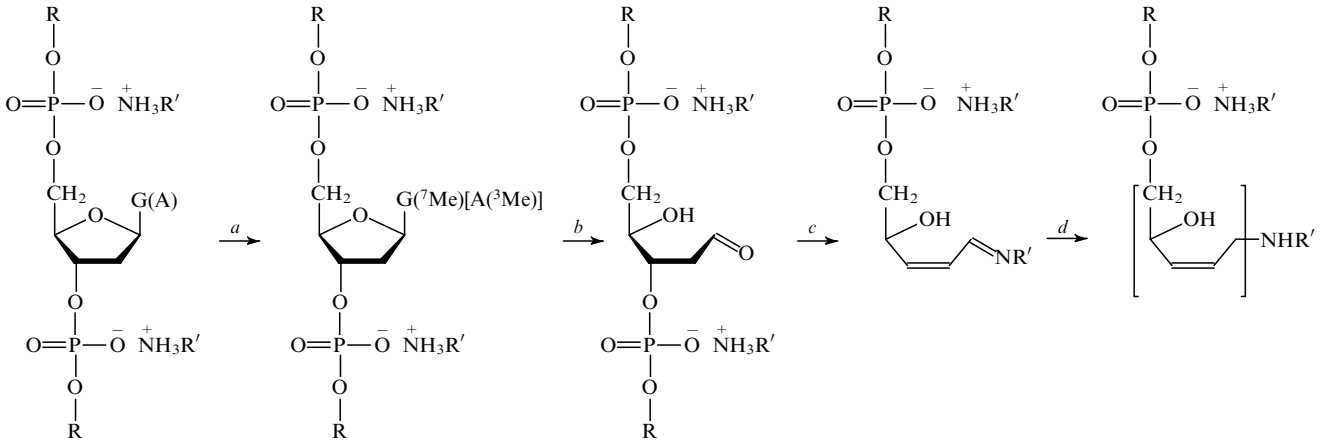
The relative arrangement of histones in the octamer was studied using cross-linking with bifunctional reagents. Such bifunctional reagent as dimethyl suberimidate, which has a large 'radius of action' (10–20 Å), cross-links practically all histone pairs within the nucleosome. This finding corroborates the theory according to which the histone octamer is the basic structural element of the nucleosome.[6–8] The use of dimethyl suberimidate has made it possible to establish the following interactions: Lys74(H2A)–Lys24 and Lys27(H2B), Lys85(H2B)–Lys115(H3) and Lys77(H4), Lys85(H2B)–Lys91(H4).[9–11] However, the radius of action of this lysine-specific reagent was too large to allow one to obtain information about the relative arrangement of histones in the core. Experiments with chemical (tetranitromethane and formaldehyde) and UV-induced cross-linking of nucleosomes gave more extensive information.[9,12–14] The strongest contacts were established between histone pairs H2A–H2B, H2B–H4 and H3–H3. UV irradiation of nucleosomes has made it possible to detect the interaction of histone H2A (Pro26) with histone H2B (Tyr37, Tyr40, Tyr42).[15] Histones H4 and H2B are cross-linked with tetranitromethane and formaldehyde as well as upon UV irradiation. The strongest contacts are localised in the C-terminal fragment of histone H2B and the N-terminal fragment of histone H4. It was thus concluded that histone H2B has two domains which interact with other histones. One of these domains localised in the C-terminal fragment interacts with histone H4, while the other one localised in the central domain interacts with histone H2A.[9] Analysis of a large body of experimental evidence on cross-linking of histones with bifunctional reagents of various lengths did not reveal a single case of histones H4–H4 interaction even where 'lengthy' cross-linking reagents were used. This prompts a conclusion that a crucial role in the formation of the $(H3–H4)_2$ tetramer is played by H3–H3 contacts. The use of carbodiimides as the cross-linking reagents has made it possible to establish the presence of H1–H2A contacts.[16–18] Cross-linking of nucleosomal chain histones with carbodiimide has shown that the 74–106 segment of histone H1 interacted with the 58–129 segment of histone H2A. Moreover, cross-links of histone H1 to all core histones, especially to histone H3, could be obtained through the use of bifunctional reagents.[19–22] It may thus be concluded that histones H3 and H2A are localised in the sites where DNA enters and leaves the nucleosomal core, which is consistent with the model of a nucleosome proposed by Mirzabekov *at al*.[23]

III. Study of DNA interactions with histones. 'Zero-length cross-linking'

The works by Mirzabekov *et al*.[23–31] have made a weighty contribution to the study of the fine organisation of nucleosomes by chemical methods. The approach suggested is based on methylation of DNA resulting in 7-methylguanine and 3-methyladenine derivatives. Comparison of kinetics of formation of 7-methylguanine and 3-methyladenine derivatives upon methylation of free DNA and DNA within chromatin revealed that histones have no effect on the alkylation of adenine residues from the side of the minor groove. However, the rate of methylation of guanine residues from the side of the major groove of chromatin DNA is by 15% lower in comparison with that of free DNA.[27–29] Determination of the length of DNA fragments in chromatin obtained by cleavage of the modified molecule at the sites of methylation revealed that histones do not protect from methylation rather extended regions of DNA in the nucleosomal chains. It was therefore concluded that histones shield the major groove of chromatin DNA uniformly, over its whole length by 15% but do not shield the minor groove. Similar results were obtained in experiments with the binding of antibiotics distamycin A and netropsin with the minor groove of DNA.[31]

The method used to determine DNA–protein interactions in chromatin is based on chemical cross-linking of definite monomeric units in the DNA molecule with amino acid residues of histones that directly interact with them. Such cross-linking (Scheme 1) involves: (*a*) labilisation of glycosidic bonds of deoxyribose residues with purine bases resulting from methylation of the latter at position 7 of guanine or at position 3 of adenine; (*b*)

Scheme 1



R is the fragment of DNA; R′ is the fragment of a protein molecule; (*a*) Me_2SO_4; (*b*) pH 6.8, 45 °C; (*c*) formation of the Schiff base; (*d*) $NaBH_4$ or $NaBH_3CN$.

cleavage of the glycosidic bond at the modified residues to yield deoxyribose fragments carrying the aldehyde groups; (*c*) formation of covalent linkages of partially depurinated DNA with the protein (in particular, the Schiff bases with the ε-amino groups of lysine provided these are nearby) followed by cleavage of the polynucleotide chain at the site of formation of these derivatives through the reaction of β-elimination and (*d*) reduction of the histone–DNA covalent bond that formed. As the covalent bond is formed directly between the deoxyribose residue of the nucleotide and the amino acid residue of the histone without any spacer, this method was named 'zero-length cross-linking'.[25, 30]

Methylation [step (*a*)] is carried out with dimethyl sulfate. Generation of a positive charge in the 7-methylguanine or 3-methyladenine fragment makes possible hydrolysis of the glycosidic bonds of DNA at the sites of modification by gentle heating at neutral pH. The deoxyribose residue that formed exists in an equilibrium between the open aldehyde and cyclic hemiacetal forms. The reaction of the aldehyde group with the neighbouring amines results in covalent addition of the DNA fragment to the histone to yield the Schiff base. The length of the cross-linked fragment is equal to the distance from its 5′-end to the cross-linking site. At low degree of DNA modification, when DNA is mostly cross-linked at only one site, the length of the cross-linked fragment indicates the position of the contact site relative to the end of nucleosomal DNA. Since the Schiff bases are rather labile, they should be stabilised by reduction with sodium borohydride or sodium cyanoborohydride to the corresponding alkylamines for further manipulations. If the reduction of the primary cross-links is carried out with $NaBH_4$, the number of cross-links formed will be close to the number of histidine residues in the protein molecule interacting with DNA. Thus, this approach is sometimes referred to as histidine cross-linking. If reduction is performed with $NaBH_3CN$ simultaneously with cross-linking, this favours the cross-linking of DNA *via* lysines. This procedure has been termed the 'lysine' protocol.[32]

The next step is sizing of the DNA fragments that formed and identification of the nature of the histones cross-linked to each of these fragments. This was carried out by two-dimensional diagonal electrophoresis, which is the second principal element of the approach proposed by Mirzabekov *et al.*[23–31] Polyacrylamide gel electrophoresis of the whole cross-linked material in the first direction was carried out under denaturing conditions in the presence of urea and sodium dodecyl sulfate (SDS). The DNA fragments cross-linked to histones are separated according to the sizes of both the DNA fragment and the protein cross-linked to them. In other words, nucleoproteins containing polynucleotide fragments of identical lengths migrate at different rates, this being the slower the larger is the size of the cross-linked histone. After treatment of the gel with pronase, which hydrolyses completely the proteins, the DNA fragments are fractionated according to their lengths in the second (perpendicular) direction. This procedure allows one to determine the lengths of these fragments using the corresponding markers. Identical fragments that had been attached to different histones appear to be displaced relative one another depending on the retardation upon separation in the first direction. On the other hand, each set of DNA fragments of different lengths which corresponds to cross-linking with the same protein is partitioned into diagonal stripes. Such DNA fragments contain no protein and can be regarded as 'protein images'. The position of the diagonal points to the relatedness of each DNA fragment of particular size to a definite histone. In most experiments, an isotope label has been introduced into the 5′-end of DNA by a conventional procedure. It consists in enzymic phosphorylation of DNA with polynucleotide kinase, which transfers [^{32}P]phosphate from [γ-^{32}P]ATP. The separated fragments are identified by autoradiography (for detailed description, see Ref. 33). It was found that the sites of DNA contacts with histones on each DNA strand are separated by a distance multiple of 10 nucleotides, which corresponds to the size of one turn of the double helix. This means that the histone octamer with the DNA wound around it interacts with one side of the double helix. Then, the opposite site is exposed to the action of DNAse I.

The arrangement of histones on both strands of the nucleosomal core DNA established by the above-described method is shown in Fig. 2.[34] Each histone forms several contacts with DNA;

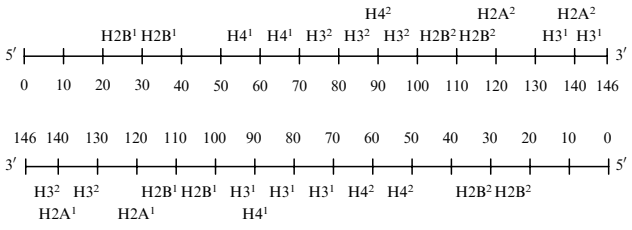


Figure 2. A symmetrical model of arrangement of histones on both strands of DNA within the core nucleosome particle. The numbers from 0 to 146 designate the distance (the number of nucleotide residues) from the 5′-end of DNA. Each of the two histone copies in a core particle is marked with a superscript 1 or 2. The histones are arranged along one strand of DNA in the following order: $H2B_{25,35}-H4_{55,65}-H3_{75,85}/H4_{88}-HB_{105,115}-H2A_{118}-H3_{135,145}/H2A_{145}$.[23, 26]

histones H3 and H4 are associated with the centre of the core particle, while histones H2A and H2B are shifted to the periphery of the nucleosomal core. The contacts between the 3′-terminal fragment of the core DNA and histones H3 have also been established. The positions of the histone dimers H2A – H2B and the histone octamer $(H3-H4)_2$ on the double-helical molecule of the core DNA are rather autonomous (Fig. 2). This is consistent with the leading role of such specific complexes in the nucleosome assembly.[35] [Presumably, nucleosomes are assembled during chromatin replication by cross-linking of the $(H3-H4)_2$ octamer to DNA to form a primary unstable nucleoprotein particle, to which the H2A – H2B dimer is then attached to stabilise the nucleosome].

Thus, the full pattern of interactions of each of the four histones with the DNA molecule within the nucleosomal core emerges. The main structural nucleosome components revealed by this approach are largely identical both in the isolated nucleosomes and within intact chromatin. The similarity of the primary organisation of nucleosomes isolated from different sources provides support for their conservative structure.[24, 25, 36]

The crucial role of histone H1 in chromatin condensation and maintenance of the 30 nm fibril, which is the next hierarchic level of chromatin organisation, was demonstrated in early studies of Georgiev *et al*.[37] The zero-length cross-linking method allowed one to establish that DNA of chromatin from *Drosophila* embryos is bound simultaneously with histone H1 and core histones.[38, 39] It is known that the linker histone H1/H5 has three structurally and functionally different domains, *viz*., a central globular domain which is evolutionarily the most conservative, a relatively short N-terminal domain and a highly basic lengthy C-terminal domain (terminal domains are the most variable parts of the histone).[40, 41] Cross-linking revealed that histone H1 interacts with the termini of the core DNA. It was found that the conservative, globular part of the H1 molecule interacts with the termini of the nucleosomal DNA and the nonstructured N- and C-terminal fragments of histone H1 interact with the external regions of the nucleosomal DNA. It was supposed that the H1 molecule serves as a bridge which binds together two neighbouring nucleosomes by folding the nucleosomal chain into a 30-nm fibril.[26]

Many polycyclic molecules having a rigid planar structure are able to intercalate, *i.e*., to be incorporated between two adjacent, stacked base pairs in the double helix of DNA. Intercalators, such as acridine, are bound predominantly to the linker site of DNA.[42] A photoaffinity reagent based on acridine, *viz*., $AcrNH(CH_2)_3N(-COC_6H_4N_3$-*p*$)-(CH_2)_3NHAcr$, where Acr is the acridine residue, was proposed for elucidation of the contacts of the linker DNA.[43] Irradiation of a complex of this reagent with a complete nucleosome results in photoaffinity modification, predominantly of proteins H1, H2A and H3. These data are in agreement with the results of binding of histone H1 with the linker DNA and corroborate the position of the other two histones on the periphery of the nucleosomal core DNA (see Fig. 2).

In its original version, the zero-length cross-linking method provided information only about the nature of the nucleotide residue involved in the interaction with one or another histone. However, a methodology of identification of amino acid residues involved in the cross-linking has been exemplified with the interaction of chromosomal DNA with histones H5[44] and H4.[45, 46] To this end, the cross-linked product was treated with nucleases or with 66% HCOOH/2% diphenylamine, after which the short oligo- or di-nucleotide fragment which remained bound to the protein was labelled with ^{32}P using polynucleotide kinase. Identification of labelled fragments was carried out by traditional methods of peptide chemistry. It was shown that modifications in histone H5 predominantly affected the histidine residues (His25 and His62) in the globular fragment of the histone molecule and the N-terminal amino group of the threonine residue (Thr1). In histone H4, the cross-linking occurred predominantly at the His18 residue localised in the central globular domain.[46]

IV. Study of functionally active chromatin

Elucidation of the mechanism of rearrangements of chromatin, which take place during its passage into a functionally active state, primarily as a result of its involvement in transcription, is of considerable importance. Identification of nucleosomes associated with regulatory and transcribed sites is carried out by the method of diagonal electrophoresis with nonlabelled DNA. Then the 'protein images' formed are hybridised with the corresponding ^{32}P-labelled probes. Heat shock genes of *Drosophila* cultured cells the heating of which to 37 °C triggers transcription of heat shock proteins (*hsp*), are studied in most details. This approach allows one to conduct parallel experiments with transcriptionally active and transcriptionally silent chromatin.[32, 47–49]

The first investigations of this kind were carried out using zero-length cross-linking approach and employed both the 'histidine' cross-linking method and the 'lysine' protocol. The results of these studies were not identical, though. In the 'histidine' method, where cross-linking of DNA to histones mostly occurs in the promoter, structural and 3′-flanking sites of heat shock activated *Drosophila* genes with the central globular protein domain, transcription evokes initial reduction of the number of core histones followed by complete removal of the linker histone H1 and, ultimately, of all histones.[47] In the case of the 'lysine' protocol, cross-linking of histones affects their terminal domains, there was no significant difference in cross-linking of active and silent chromatin. It was concluded from these data that activation of transcription did not result in complete removal of histones from the transcriptionally active site of chromatin with the exception of the promoter site where histone – DNA interactions are practically absent (at least the sites in the promoter region with the length of up to 200 b.p. are free from histones). With further progress of transcription, the interactions of central globular domains of histones with DNA are weakened but the contacts of histone N- and C-terminal fragments with DNA are preserved.[32] Thus, the passage from transcriptionally silent to transcriptionally active chromatin is accompanied by loosening or unfolding of the nucleosome structure, *i.e*., significant structural rearrangements take place.

More recent studies made use of UV irradiation of whole cells for cross-linking of DNA to proteins. Hybridisation with 'protein images' was carried out with DNA probes which represent a promoter fragment of *hsp*70 gene DNA, a fragment with the length of 900 b.p. within the transcribed subunit of the *hsp*70 gene and a nontranscribed fragment of ribosomal DNA. It was found that interactions of histone H1 with DNA occur even under conditions of active transcription. The exception is the promoter region where DNA – histone H1 contacts are absent. At the same time, in the case of active chromatin the promoter site interacted with two additional proteins with molecular weights of 50 and 100 kDa. Probably, these proteins are the factors responsible for the regulation of transcription. The 100 kDa protein binds predominantly to the encoding strand, whereas the 50 kDa protein binds to the complementary noncoding strand.[49] It should be noted that cross-linking of the promoter to these two proteins takes place only upon activation of the heat shock gene and is absent in the inactive gene. It was shown[50] that the set of histones modified with azidoarylbisacridinylspermidine and cross-linkable to the active fraction of chromatin which is preferentially split with nucleases is different from that of the total preparation of chromatin. In the active fraction of chromatin, only histone H1 is subject to photoactive labelling, whereas histones H2A and H3 are not. This finding seems to corroborate the conclusion that DNA in the active fraction of chromatin is less tightly bound to the histone core and the active chromatin has a more loose structure.

Among nonhistone proteins of chromatin, HMG proteins with high electrophoretic mobility are studied in most details. HMG14 and HMG17 (10 000 and 9247 kDa, respectively) have been defined as proteins of transcriptionally active chromatin.[51]

These proteins are known to bind to nucleosomes, this binding being especially tight in the case of active chromatin. Core nucleosomes and nucleosomes containing histone H1 were isolated from chicken erythrocyte chromatin, reconstituted with HMG14/17 proteins and then the proteins were cross-linked to DNA.[52] Analysis of the cross-linked material by two-dimensional gel electrophoresis revealed that cross-linking of HMG14/17 to nucleosomes does not influence the distribution of histones along the DNA strand either in the core particle or in the complete nucleosome. The main contact sites of these proteins with DNA are at a distance of 25 and 115 nucleotides from the 5′-end of the core particle. The contacts of the core histones H2A, H2B and H3 are grouped in the same terminal fragment of core DNA (see Fig. 2). These data are consistent with the results of cross-linking of proteins HMG14/17 and core histones to the lysine-specific bifunctional reagent, 2-iminothiolane.[53] The photoactivated reagent, *N*-hydroxysuccinimide 3-[(4-azidophenyl)dithio]propionate (SADP), cross-linked HMG proteins to the central fragment of histone H3.[54] The proteins isolated from cell suspensions of chicken blood were used.[54] The radioactive label was introduced into the proteins by reductive alkylation with $[^{14}C]H_2CO$ after which the proteins were derivatised with SADP. Acylation at lysine residues converted the individual proteins HMG14 and HMG17 into their photoactive analogues carrying the $N_3C_6H_4SS(CH_2)_2C(O)$ fragment at amino groups of several lysine residues. UV irradiation of a complex with nucleosomes generated nitrene from the azide residue, which was cross-linked to one of the neighbouring proteins. After separation of the cross-linked proteins, the disulfide bridge can be cleaved by disulfide exchange with mercaptoethanol, which facilitates identification of histones cross-linked to HMG proteins. A more lengthy reagent, *viz*., *N*-hydroxysuccinimide 3-[(1-(2-nitro-4-azidophenyl)-2-aminoethyl)-dithio]propionate (SNAP), preferentially cross-links HMG14/17 to histone H2A.[55] Probably, owing to differences in the geometry and size of SNAP and SADP molecules, they interact through their hydrophobic and photoreactive termini with different groups on the histone core surface where histones H2A and H3 are in close proximity to each other. These results suggest that the HMG fragment cross-linked to histone H3 is close to the Cys110 residue of histone H3 localised on the nucleosome surface. The Cys110 residue is in the open state; its conformation is characteristic of transcriptionally active chromatin.[56] This finding prompts a conclusion that cross-linking of HMG14/17 proteins to the nucleosome may strengthen this conformation.

Experiments with Ehrlich ascites cell nuclei fixed with formaldehyde and subjected to DNA – protein cross-linking were undertaken to study the distribution of histones in the *de novo* synthesised DNA strand. The latter was synthesised in the presence of 5-bromodeoxyuridine 5′-triphosphate. Thus, it contained 5-bromodeoxyuridine residues and could therefore be identified and investigated using polyclonal antibodies against 5-bromodeoxyuridine. It was shown that histones of *de novo* synthesised chromatin had the same stoichiometry as the original one. Histone H1 binds to the daughter DNA strand simultaneously with, or immediately after, the cross-linking of core histones, although the cross-linking to the *de novo* synthesised DNA is weaker in comparison with the original chromatin.[57]

An important role in the cross-linking of transcription factors to nucleosomes is played by specific proteins, *viz*., nucleoplasmin, or its yeast analogue, NAP-1 (nucleosome assembly protein). These proteins induce the formation of a ternary complex of the transcription factor (nucleoplasmin or NAP-1). The use of a chemical approach based on cross-linking of histone octamer proteins with dimethyl suberimidate confirmed the fact that the mechanism of action of these proteins consists in elimination of the H2A – H2B dimer followed by elimination of the remaining histone tetramer $(H3 – H4)_2$. This study made use of the yeast activating factor GAL4-AH, which binds to a definite DNA sequence preceding the lacZ gene. This factor was investigated with the help of a specially designed system which included a DNA fragment (153 b.p.) and a binding site for GAL4 separated by a distance of 35 b.p. from one of the ends of the nucleosomal core. It was found that the stimulating effect of nucleoplasmin or NAP-1 (which under normal conditions stimulate the binding of GAL4-AH to native nucleosomes) was not manifested when nucleosomes with histone octamers cross-linked with dimethylsuberimidate were used. However, after reconstitution of nucleosomes from the cross-linked H2A – H2B complex with subsequent introduction of all the other core histones, the functions of nucleoplasmin and NAP-1 were not disturbed. Thus, separate removal of the first pair of histones H2A – H2B from the remaining part is problematic.[58] According to previous reports, such removal may not be complete but rather result from significant bond loosening.

Mapping of DNA – protein contacts along definite sequences of DNA was carried out using a model of ribosomal genes. This model is very convenient for establishing the relationship between the regulation of gene activity and structural rearrangements in chromatin.[59, 60] Ribosomal RNA genes are present in *Drosophila* cells as 250 copies where they are grouped into tandem repeats in which transcription elements are separated by spacers containing repeating sequences (239 b.p.) flanked by recognition sites for AluI restrictase. Restriction enzymes (restrictases) catalyse the cleavage of DNA at tetra- or hexa-nucleotide sequences which are strictly specific for each restrictase. These repeating sequences are homologous to the promoter sites of ribosomal genes and play a prominent role in their transcription. UV-induced cross-linking and hybridisation with 'protein images' allowed one to identify two proteins (mol. masses 50 and 70 kDa) associated with the Alu-repeat.

V. Study of chromatin using reactive oligonucleotide derivatives

Studies of affinity modification of chromatin with various oligonucleotide derivatives are of interest as an approach to the investigation of the structure of this most important type of DNA – protein complexes as well as due to the necessity of elaboration of methods for targeted attack on definite genes and mRNA with complementary oligonucleotides aimed at modulating their expression and induction of directed mutagenesis. This approach was first proposed by Grineva *et al*.[61] and exemplified with reactive oligonucleotide derivatives in order to direct (address) a bound reactive group to a definite site in the target nucleic acid with the help of a complementary oligonucleotide. Hence, the original name of this method is 'complementarily addressed modification'. At present, the methods based on the reactions of definite sense sites of a target nucleic acid with specific oligonucleotides, their derivatives or analogues are referred to as the antisense approach. This approach is gaining popularity as a helpful tool in molecular-biological studies and the reagents are considered as potential therapeutically valuable compounds, in the first place, antiviral and antitumour preparations which interact with genetic structures.[62, 63]

In the majority of cases, antisense oligonucleotides are used for cross-linking to single-stranded (matrix or viral) RNA. The possibility to act upon DNA by suppressing the effects of oncogens or integrated viral genomes would significantly extend the range of their therapeutic applications. Therefore, DNA presents an attractive object for application of such oligonucleotide derivatives.

In this connection, a great deal of effort is given to the design of oligonucleotides able to suppress the DNA function due to the formation of ternary complexes.[63] However, an alternative approach consisting in the treatment of unwound regions of DNA that exist in chromatin with oligonucleotide derivatives is also possible.

Chromatin DNA of eukaryotic cells is not a strictly double-helical structure over its whole length. In the course of cell functioning (*e.g*., during replication or transcription), definite

sites of DNA can be temporarily unwound. The DNA of complex nuclear structures may contain single-stranded regions which can interact with oligonucleotide derivatives. Such single-stranded sites of the DNA molecule can be formed as a result of DNA interaction with specific proteins able to unwind the double helix or due to the presence of superhelical domains in DNA. The possibility of specific chemical modification of DNA within chromatin and in the intact nuclei with complementarily addressed reagents was experimentally studied at the Novosibirsk Institute of Bioorganic Chemistry of the Siberian Branch of the Russian Academy of Sciences (see below).[64–68]

Repeating sequences, which are abundant in the eukaryote genome and represent a fundamental distinction of the genome organisation in higher eukaryotes, were selected as targets to study the interaction of oligonucleotides with chromatin DNA.[69] The human genome contains short (about 300 b.p.) repeats which alternate with unique sites and contain a site recognisable by restrictase AluI.[70] These repeats are referred to as Alu-repeats and constitute about 3% of the human genome (3×10^5 copies per genome). The left and the right halves of the Alu-repeat are characterised by a high degree of homology and are flanked with oligoadenylic regions (up to 20 b.p. in length). The dinucleotide repeat $(dG\text{-}dT)_n$ with its complementary oligonucleotide $p(dA\text{-}dC)_6$ comprises about 0.5% of the eukaryotic genome. The occurrence of relatively short (up to 50 b.p.) sites of this repeat occur in the eukaryotic genome in 1 per 30 000–100 000 base pairs, on the average.[71,72] According to numerous reports, the $(dG\text{-}dT)_n$ repeat was detected in the sites of transition of DNA from the *B*- to the *Z*-conformation.[73–75] Such a transition may provoke local unwinding of DNA in these sites of chromatin and thus make them accessible to complementary interaction with the corresponding oligonucleotide derivatives, *e.g.*, $p(dT)_{16}$, $p(dA)_{16}$, $p(dA\text{-}dC)_6$ and pd(TTCTCCTGCCTCAGCCTCTT) (which is complementary to the most conservative site of the Alu-repeat family).

In the first series of these investigations, oligonucleotide derivatives, *e.g.*, $p(dT)_{16}$, were used containing a reactive alkylating group, *viz.*, 4-[(*N*-2-chloroethyl-*N*-methyl)amino]benzylamine attached through a phosphamide bond to the 5′-terminal phosphate group. The experiments were carried out on chromatin isolated from rodent and human cells as well as on metaphase chromosomes and intact nuclei.

The study of a possibility of specific cross-linking of an oligothymidylate derivative, complementary to the polyA-repeats of DNA widely distributed in the eukaryotic genome, to DNA from the soluble fraction of human placenta chromatin demonstrated that the alkylating derivative modifies chromatin at room temperature.[64] Nonmodified $p(dT)_{16}$ strongly inhibits the modification. At the same time, the oligonucleotides which are not complementary to the polyA-repeats do not influence the modification, which points to the specificity of interaction. Preliminary treatment of chromatin with RNase A under conditions ensuring hydrolysis of polyriboadenylate sequences present in the majority of chromatin matrix RNAs does not virtually exert any influence on the degree of modification with the same reagent. Hence, it is DNA that is subjected to modification.

It was shown that reactive oligonucleotide derivatives can specifically modify DNA both within chromatin and in the most densely packaged deoxyribonucleoproteins, *viz.*, metaphase chromosomes. However, nuclear DNA was not subjected to modification under identical conditions.[65,76]

In the case of modification of the Alu-repeat, the specificity of modification was additionally confirmed by data from restriction analysis. An oligonucleotide selected for modification was complementary to one of the most conservative sites of the Alu-repeat localised inside the 170 b.p. fragment cut out with Sau3A restrictase. After modification of human HeLa cell chromatin, DNA was isolated and cleaved with restrictase Sau3A. The resulting fragments were separated by electrophoresis in polyacrylamide gel. Autoradiography of the gel revealed that the radioactivity imparted by the covalently attached oligonucleotide derivative was detected in the 170-b.p. fragment, whereas in control experiments where the oligonucleotide derivative had a different sequence, the radioactivity was not found.[77]

It was shown that preliminary treatment of chromatin preparations with S1-nuclease, which cleaves under mild conditions single-stranded regions of DNA with preservation of the general morphology of these specimens, results in practically complete inhibition of chromatin modification with oligonucleotide derivatives.[76,77] This indicates that the interaction of oligonucleotide derivatives with DNA occurs *via* the formation of their complementary complexes with unwound regions of chromatin DNA.

A study of the interaction of the alkylating oligothymidylate derivative with chromatin isolated from various types of cells (*e.g.*, replicating cells of regenerating rat liver and chicken erythrocyte cells in which chromatin was completely repressed both in transcription and replication) made it possible to gain insight into the nature of structural peculiarities of DNA in chromatin that are responsible for the formation of local unwound regions allowing for specific interaction with oligonucleotide derivatives.[67,68]

Regenerating rat liver is a classical object for investigation of various processes occurring in the cell during intensive replication. The maximum replication in liver cells is observed 22–24 hours after partial hepatectomy. The nuclei isolated from intact and regenerating rat liver cells were treated with the $p(dT)_{16}$ alkylating derivative. The relative degrees of modification of DNA appeared to be identical in both cases. This suggests that the formation of replication forks does not cause any significant increase in the number of polydeoxyadenylate sites accessible to the complementarily addressed reagent. Similar results were obtained in the studies designed to compare the degree of modification of DNA at polydeoxyadenylate sequences in the nuclei of both non-synchronised cells and cells synchronised in the S-phase of the cell cycle where DNA synthesis occurs with the greatest intensity.

Differentiation of erythroblasts into erythrocytes is accompanied by inhibition of replication and transcription. Fully repressed (both in transcription and replication) chicken erythrocyte chromatin is modified at poly(dA) sequences with the same efficiency as does chromatin isolated from regenerating rat liver. This suggests that activation of replication and transcription does not increase the accessibility of poly(dA) sites of DNA to complementarily addressed modification.[68]

It has been shown that the presence of a nuclear membrane is not an obstacle to the penetration of the oligonucleotide inside the cell. The degree of modification of DNA in both intact nuclei and the 'suspension' chromatin obtained by mechanical disintegration of the nuclear membrane are nearly identical. Therefore, it would be logical to suppose that the existence of unwound sites of DNA containing the targets is most likely determined by the level of superhelisation of DNA and the structural and functional characteristics of chromatin.

It is known that in contrast with suspension chromatin and intact nuclei, the soluble fraction of chromatin in a buffer containing no bivalent metal ions is largely represented by unwound nucleosome strands. The degree of modification of DNA of the 'soluble' fraction was one order of magnitude lower than that of the 'suspension' chromatin and intact nuclei.[76] This result may be due to the presence in the latter of loop-shaped structures formed by DNA fibrils fixed on the nuclear matrix (which enhances superhelisation of DNA) and nonhistone proteins.

Evidence for the role of superhelisation of DNA in addressed alkylation of chromatin can be also derived from the dependence of the relative degree of DNA modification in intact nuclei on ionic strength. An increase in NaCl concentration up to 0.15–0.2 M is accompanied by a decrease in the degree of modification of DNA.[67] In this concentration range, the activity

of endogenous topoisomerase I, which favours relaxation of the superhelical conformation of DNA, is at maximum.

The effect of changes in chromatin structure caused by removal of definite protein groups on the degree of accessibility of DNA for affinity modification was studied in experiments with deoxyribonucleoproteins DNP-0.35, DNP-0.6 and DNP-2.0 obtained from chromatin by treatment with 0.35, 0.6 and 2.0 M NaCl, respectively.

It is known that treatment of chromatin with 0.35 M NaCl yields a fraction of nonhistone proteins containing HMG-proteins. Removal of these proteins from HeLa cell chromatin decreases the relative degree of modification of poly(dA) and $(dG\text{-}dT)_n$ sites of DNA by addressed reagents.

Treatment of chromatin with 0.6 M NaCl results in the removal of histone H1 and some nonhistone proteins. The degree of modification may increase under these conditions because the removal of histone H1, which binds to the linker sites of chromatin DNA, results in their liberation. It is these sites that are potentially more accessible to complementarily addressed modification with the chloroalkyl derivative. Treatment of chromatin with 2 M NaCl results in the removal of the majority of nonhistone proteins and of all histones. The relative degree of modification of poly(dA) and $(dG\text{-}dT)_n$ sites of DNA decreases, but still markedly exceeds that of isolated DNA. Electron microscopy of DNP-2.0 obtained by such treatment revealed DNA fibrils as loops fixed on the nuclear matrix. Our experimental results may testify to the contribution of specific chromatin proteins preserved in the structure of chromatin after its treatment with 0.6 M NaCl but completely removed by treatment with 2 M NaCl to the appearance of sites in DNA that are accessible for modification and enriched with poly(dA)- and $(dG\text{-}dT)_n$-repeats.

Among alkylating oligonucleotide derivatives, that with a cholesterol residue at its 3′-end manifested the greatest efficiency for modification of DNA from intact nuclei which exceeded that of the oligonucleotide derivative devoid of cholesterol residue by an order of magnitude.[78]

Later it was shown that the alkylating oligonucleotide derivatives modify not only DNA when they form specific complexes with chromatin DNA, but also definite proteins, presumably localised at the site of their interaction with the DNA 'target'.[76,77,79]

Despite intensive investigations into the structure of metaphase chromosomes and interphase chromatin, the topography of chromosomal proteins is still poorly examined. Most often, specific binding of proteins to definite sequences of DNA is carried out by gel filtration or affinity chromatography on columns with immobilised DNA. However, the conditions for complex formation provided by these methods do not strictly simulate the conditions inside the cell. Moreover, they do not reflect the dependence on the DNA conformation stabilised by superhelisation or DNA–protein interactions. Therefore, the use of these methods for the study of dynamic changes in the protein environment of DNA sequences in chromatin and regulatory processes is limited. In this context, addressed modification may become promising in the study of protein environment of DNA sequences in the unwound regions of chromatin.

The chromatin proteins subjected to specific modification with oligonucleotide derivatives that are complementary to poly(dA)-, $(dG\text{-}dT)_n$- and Alu-repeats were analysed by gradient SDS electrophoresis. Protein specimens were treated with micrococcal nuclease to destroy the oligonucleotide fragment covalently attached to the protein. This fragment might change the mobility of the modified protein in electrophoresis.

It has been shown that modification with the $p(dT)_{16}$ alkylating derivative affects at least two major proteins in intact rat liver nuclei with average molecular weights of 19–20 and 27–28 kDa.[79] Addition of a 10-fold excess (with respect to the reagent) of nonlabelled $p(dT)_{16}$ results in inhibition of alkylation of these proteins. However, an excess of the oligonucleotide pd(CATGCAAAACCTTCCC) having the same length but a random nucleotide sequence does not interfere with the protein alkylation, which points to the specificity of the interaction of the reagent with the proteins. Such interaction may occur in both the complementary complexes of the oligonucleotide with the target DNA and with proteins showing affinity for oligothymidylate. Analysis of the chromatin protein components alkylated with $p(dA)_{16}$ and $p(dT)_{16}$ derivatives revealed them to be the same in both cases. These data suggest that this reaction occurs through the formation of a complementary complex between the oligonucleotide address of the reagent and the corresponding sites of the unwound chromatin DNA.

Interestingly, the protein with the molecular weight of 19–20 kDa is modified with the reagent within both intact chromatin and the DNP complex prepared by treatment of chromatin with 2 M NaCl (DNP-2.0). Hence, this protein can be related to the group of nuclear matrix proteins.[79]

Alkylation of nuclei with the $(dA\text{-}dC)_6$ derivative yields a radically different set of modified proteins. Six proteins (or groups of proteins) are specifically modified with the $p(dA\text{-}dC)_6$ derivative. An oligonucleotide having the same length but a different sequence has no effect on their modification.

Treatment of HeLa cell nuclei with the $p(dA\text{-}dC)_6$ derivative gave a more complex set of modified proteins.[76] In addition to proteins presumably analogous to modified proteins from rat liver (19.5, 21, 25.5, 31, 33 and > 66 kDa), modification affects a number of other proteins the alkylation of which is not inhibited by an excess of the oligonucleotide. Since inhibition of modification with an excess of the oligonucleotide of the same sequence does not rule out the possibility of alkylation of proteins that are not associated with local unwinding sites but show affinity for the address oligonucleotide, cell nuclei were treated with S1-nuclease (under conditions of cleavage of single-stranded regions of chromatin DNA) to demonstrate the specificity of modification of the proteins in the complementary complex. This study showed that seven proteins presumably localised in local unwinding sites were subjected to specific modification.[76]

Several researchers have demonstrated[80–83] that natural polyamines (spermine and spermidine) induce the transition of DNA from the *B*- to the *Z*-conformation and stabilise the left-helical *Z*-conformation under physiological ionic conditions. The affinity modification of chromatin and intact eukaryotic (human and rat liver) cell nuclei with the $p(dA\text{-}dC)_6$ alkylating derivative at the $(dG\text{-}dT)_n$-repeats of DNA has been studied under physiological conditions and at high concentrations of spermine and spermidine.[84] Analysis of chromatin proteins subjected to specific modification with this oligonucleotide derivative revealed that the efficiency of modification of both DNA and the proteins increases in the presence of high concentrations of spermine and spermidine and sharply decreases after preliminary treatment of chromatin with S1-nuclease under conditions of mild hydrolysis of single-stranded regions of DNA. These data testify to the fact that the $B \rightarrow Z$ transition is a reason for the presence of unwound sites accessible for the interaction with a complementary oligonucleotide in the DNA containing the $(dG\text{-}dT)_n$ repeating units.

Modification of HeLa cell nuclei with the $p(dT)_{16}$ derivative affects four proteins (19, 47, 61 and > 66 kDa). Their modification is inhibited by an excess of the nonmodified oligonucleotide and by preliminary treatment with S1-nuclease.

Treatment of cell nuclei with the alkylating derivative of pd(TTCTCCTGCCTCAGCCTCTT), which is complementary to the most conservative site of the Alu-repeat, allowed one to identify a number of proteins that are modified apparently during the formation of a complementary complex between the oligonucleotide address and the unwound regions of chromatin DNA. Data from modification with an excess of the free oligonucleotide and its inhibition upon prior treatment with S1-nuclease testify to specific modification of proteins or groups of proteins with molecular weights of 18, 22, 26, 28, 43, 68 and 93 kDa and of two proteins with molecular weights > 94 kDa.

The transition of chromatin from the interphase state to metaphase chromosomes is accompanied by significant structural rearrangements resulting in structures with the maximum level of DNA compactisation. The use of reactive oligonucleotide derivatives for modification of interphase nuclei and metaphase chromosomes clearly demonstrates that the sets of specifically modified proteins for these two chromatin states differ essentially. Thus, the 28 kDa protein is not subjected to modification within chromosomes, while proteins with molecular weights of 27, 31 and 40 kDa, which are not modified within the interphase nuclei, do undergo modification .

Comparison of the sets of the alkylated proteins isolated from the nuclei of intact and regenerating rat liver cells has demonstrated the presence in the latter of two additional modified proteins with molecular weights of 22–23 and 29–30 kDa along with 19–20 and 28–29 kDa proteins.[79]

The differences in the sets of modified proteins can be explained either by changes in their accessibility for alkylation due to the structural rearrangements resulting from activation of chromatin during replication or by changes in the protein environment in the poly(dA) sites of DNA.

In order to make sure that the given set of proteins is not directly related to the chemical nature of the reagents used for their modification, analogous experiments were carried out with $p(dT)_{16}$ derivatives. In this study, photoactivated fragments carrying an azido group, instead of the alkylating group, *viz*., $XC(O)NH(CH_2)_2NH$, where X = 2-NO_2-5-$N_3C_6H_3$, 4-$N_3C_6H_4$ and 4-$N_3C_6F_4$, have been attached to the 5′-ends. The set of modified proteins was practically identical in both cases. The degree of DNA modification with photoactivated reagents was lower, whereas the degree of protein modification was much higher than with the alkylating reagent. In all cases studied, the modification was specific, *viz*., oligothymidylate, which contained no reactive group, strongly inhibited this process.[85]

Recently it has been established that reagents with a *p*-azidoaniline residue as a photoreactive group stand out among other commonly used photoactivated reagents in terms of specificity. Such reagents have practically no effect on the nucleotide components.[86] Therefore, the spectrum of proteins modified by the oligothymidylate derivatives carrying a photoreactive arylazido group, 2-NO_2-5-$N_3C_6H_3C(O)NH(CH_2)_2NH(pdT)_{16}$ (**1**) and 4-$N_3C_6H_4NH(CH_2)_4NH(pdT)_{16}$ (**2**), have been compared.[87] Photomodification of intact nuclei isolated from HeLa cells has shown that the degree of modification with the reagent carrying a *p*-azidoaniline residue (**2**) is much higher, while the set of modified proteins is practically identical.

In the same study,[87] the authors compared the sets of proteins photomodified with reagent **1** within the nuclei isolated from HeLa cells and synchronised at the G_1/S interface and in the S-phase. In this case, the spectra of modified proteins were different, *viz*., the 160 kDa protein was present only in the nuclei isolated from cells synchronised at the G_1/S interface. This result points to the possibility of usage of photoreactive oligonucleotide derivatives for the analysis of changes in the protein environment in regions containing unwound DNA sequences and for the study of their accessibility in different stages of the cell cycle.

A $p(dT)_{16}$ derivative which contains simultaneously an alkylating group (ClX) at the 5′-end and a biotin residue (Bio) at the 3′-end of the oligonucleotide [ClX-$p(dT)_{16}$-Bio] has been synthesised in order to visualise the sites of cross-linking of oligodeoxyribothymidylate to chromatin.[88] Biotin manifests extremely high affinity for streptavidin and its conjugates. It was demonstrated that the introduction of the biotin residue into the 3′-end of the alkylating derivative of the oligonucleotide does not influence its reactivity. The efficiency of DNA modification with both ClX-$p(dT)_{16}$ and ClX-$p(dT)_{16}$-Bio is nearly identical. This fact was used to visualise the modification sites by luminescence microscopy. The study was carried out on HeLa cells and on cells of hybrid clones containing human and Chinese hamster chromosomes.[89] Modified cells were treated with the streptavidin–fluorescein isothiocyanate conjugate.

The specific luminescence of nuclei after treatment of hybrid cells was of two types. The luminescence was the most intense in the nucleoli. However, in some cells point luminescence was observed in the sites that are not directly linked with the nucleoli but are discretely distributed over the nucleus volume. This suggests that the regions of active transcription of chromatin (nucleoli) are subjected to the most intense modification, whereas the densely packaged DNA of heterochromatin is inaccessible.

Modification of HeLa cell chromatin has demonstrated that in these cells the common signal elicited by the nucleus is much higher in comparison with hybrid human–hamster cells, apparently as a result of intensive proliferation of HeLa cells. In addition, the genome of a hybrid cell is partly inactivated. Apart from nucleolar and point signals, HeLa cell nuclei display a third type of specific luminescence, namely the luminescence of the near-membrane region of the nucleus where polyA sites are especially abundant.

We have developed[89] a procedure for visualisation of modification sites in chromatin which is based on *in situ* hybridisation but differs essentially from it. In the case of standard technologies, the preparations used for hybridisation are first fixed to preserve the morphology, after which DNA is denatured for further hybridisation with the probe. This method involves specific modification of a target sequence of DNA within the native chromatin or intact nuclei, which affects only those sequences of DNA that are localised in the sites of its local unwinding.

VI. Conclusion

In-depth studies of chromatin by chemical methods based on molecular resolution and registration of the dynamic events are still in the initial stage. However, one can see from the above data that chemical approaches make it possible to establish the fine structure of nucleoprotein complexes within chromatin, to identify even the interacting amino acid residues in the case of protein–protein contacts and the interacting amino acid and nucleotide residues in the nucleoproteins. A methodology has been developed for establishing these contacts for one of the most essential functional processes, *viz*., the transition of chromatin into a transcriptionally active state. There is no doubt that these approaches will gradually progress to hold a central position in the study of other vital processes, such as replication, repair and recombination.

Another important feature of chemical methods is the possibility to detect new components of nucleoprotein complexes as can be evidenced from the discovery of two novel proteins interacting with the promoter sites of the hsp gene. At present, separation of hundreds of proteins by two-dimensional electrophoresis does not present any problem. However, establishing of physical and functional contacts between individual proteins and between proteins and nucleic acids (especially those localised directly in the intact nucleus) is a task which can be successfully solved through the use of chemical approaches.

Finally, the changes in the spectrum of protein contacts observed during activation of transcription in chromatin and interactions of oligonucleotides with proteins in different phases of the cell cycle provide evidence that chemical approaches offer new possibilities for recording the changes that take place during transitions from one state of chromatin to another.

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