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STATISTICAL ANALYSIS OF DATA ON LINKER HISTONES/DNA INTERACTIONS

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Linker histones (H1, H1o H5, subtypes and variants) play a pivotal role in formation of higher order chromatin structure and thus – as main regulators of the expression of genetic information kept in DNA. That is why the knowledge of the nature of linker histones/DNA interactions is of a greatest interest in understanding of such important issues as transcription regulation, cell division, and cancerogenesis. As DNA is a main "target" of most anticancer antibiotics, the analysis of competitive reactions between that drugs (in our case actinomycin D and netropsin) and linker histones for binding to certain sites in DNA gives hopeful information concerning the mode of such interactions. In this work we present statistical analysis of some experimental data concerning the influence of some anticancer antibiotics on linker histones/DNA interactions. First, it was investigated the formulated hypothesis of the dependence of H1/DNA interaction on actinomycin D concentration. Such a relation was expected knowing the different mode for binding of the both drugs to DNA double helix. The applied statistical analysis using chi-square test for independence showed that the concentration of Actinomycin D in reaction mixture had no essential effect on linker histone/DNA binding. On the contrary, the same analysis with the second antibiotic – netropsin showed that we could not reject the hypothesis of dependence. Some other statistical models are also proposed, applying χ^2 test for homogeneity, test of Willcockson, Smirnov's test and others.

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1. Introduction

The organization of DNA in eukaryotes in compact structures (chromatin) is due to the nuclear proteins including linker histones – H1, H1o, H5, and subtypes (van Holde, 1987; Zlatanova, 1990; Zlatanova and Yaneva, 1991a; Jerzmanowski, 2004) demostrated in Fig.1.

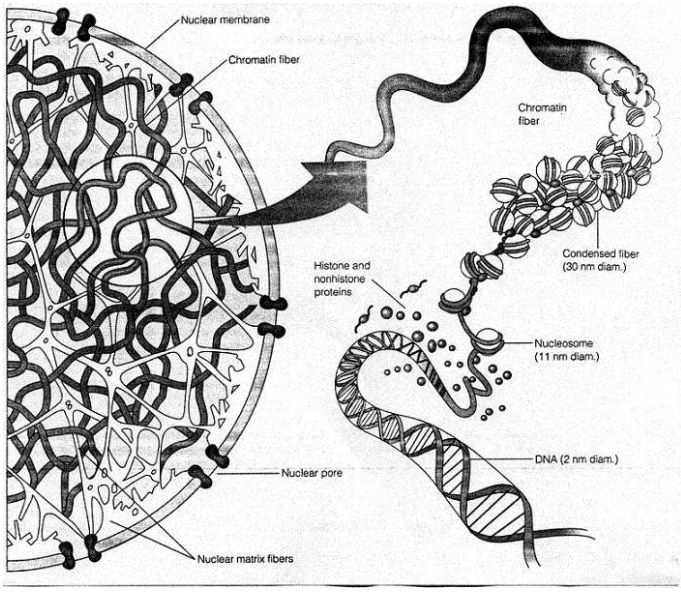

Figure 1: Levels of DNA organization in eukaryotic cell: double helix → super helix → nucleosomes → solenoid → chromosomes. The participation of linker histones (H1) in formation of higher-level chromatin structure is obvious (according to Mattews and van Holde, 1995).

The comprehensive data for position of linker histones in nucleosomes and in the compact forms of nucleosomal filaments have been interpreted as indicative of its critical function in chromatin architecture and dynamics and consequently in the regulation of such fundamental processes as transcription and chromosome condensation (Zlatanova and Leuba, 2004). Recently Zlatanova, Caiafa and van Holde (2000) reviewed the evidences for involvement of linker histones in transcriptional regulation and proposed an original scenario according which the reversible and controlable binding and displacement of these class proteins determine the accessibility of DNA from chromatin to the transcriptional machinery and further processing. The way linker histones interact with DNA – major or minor groove, sequential and conformational preferences, and the active parts of

protein molecule – are the object of versatile investigations by many outstanding laboratories (reviewed by Churchill and Travers, 1991; Zlatanova and Yaneva, 1991b; Zlatanova and van Holde, 1996; Mamoon et al., 2002). Competitive reactions with some anticancer antibiotics throw light to the mode of linker histones/ DNA interactions, and also to the mechanism of action of these antibiotics, targeting double-helix DNA molecule (Burckhardt et al., 1983; Copenhaver et al., 1995; Wemmer, 1998; Yaneva et al., 2000). Due to the essential for realization of genetic information interaction of DNA with regulatory proteins, during the past decade a hypothesis for existence of "two additional channels of information in DNA" arose – of major and minor groove (Lown, 1994)- see Fig. 2. The major groove is exploited mainly by control function proteins – promoters, repressors, enhancers; the minor groove is attacked by different enzymes, proteins with regulatory and architectural function and antibiotics, all known as minor-groove binders – MGB (Brosh et al., 2000). Thus the minor groove seems to be much vulnerable to attacks structure. This is considered to be an important reason for evolution of antibiotics - to attack directly DNA of concurrent organism (Neidle et al., 1987; Kahne, 1995; Jeeninga et al., 1998; Moravek et al., 2002).

Actinomycin D (Act. D) is a wide-spectrum antibiotic, naturally produced by some species from Streptomyces genera. The drug structure has been intensively investigated and determinated by means of chemical and physical methods (reviewed by Neidle at al., 1987; Pullman, 1989; Bailly et al., 1994; Chow et al., 2002; Hou et al., 2002). Its structure includes a flat phenoxazone ring and two circle lactopeptones attached on both sides of the molecule Fig.3). In spite of the fact that Act. D has been used as a successful anticancer drug since 1972, its mechanism of action was elucidated only ten years ago (Bailly et al., 1994). Recently the "shuffling hypothesis" was proposed by Fox and Waring (1986). According to it the antibiotic molecule initially binds accidentally to certain regions of DNA and its phenoxazone ring penetrates inside the double helix, intercalating between two planar bases (preferentially GC). If it happened to be its "favorite" binding site, its two peptide lactones "invade" into the minor groove from both sides. Otherwise, the drug molecule moves along the DNA chain and "shuffles" finding the preferred site.

2. Experimental data on drug-competition assays and hypotheses

Our work is based on a series of experiments of competitive reaction between Act. D and linker histone H1 for binding to DNA double helix (drug-competition assay). The existing experimental data concerning linker histones/DNA inter-

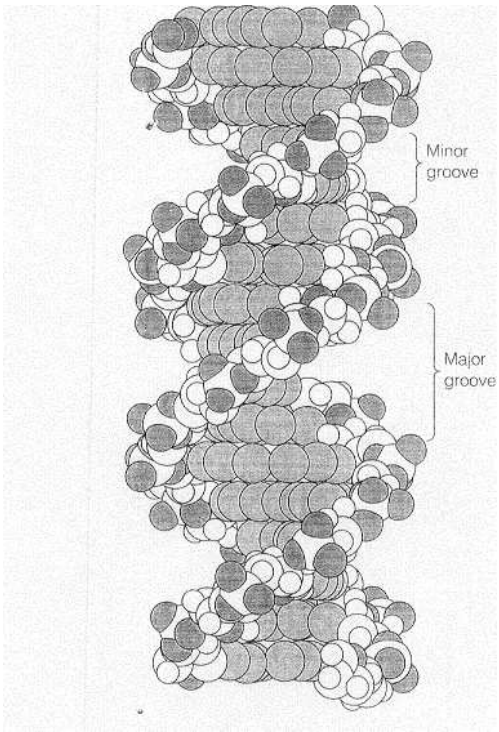

Figure 2: Computer drawing of DNA double helix: the minor and the major grooves of the polynucleotide chain might be clearly recognized (designated on right).

actions (with major or minor groove) are quite contradictory. We have studied the association of H1 with DNA in competitive reactions with Act.D – as a function of its final concentration in the reaction mixture. The main approach in this research was the observation of the mobility changes of DNA molecule (alone or complexes with proteins) in electric field – electromobility shift assay (EMSA). As a DNA probe an isolated discrete fragment of 2073 base pairs from plasmid pUC19 digested with restriction endonuclease Bst NI was used. It has been incubated with histone H1 in the presence of increasing concentrations of the antibiotic (the protein/DNA ratio was constant – about 2.0 w/w). In such a reaction the antibiotic might has facilitating (F), inhibiting (I) or no effect (N) on the histone/DNA complex formation. An important difference appears performing the experiment with preliminary incubation of the drug with DNA and then consequent addition of histone to the incubation mixture. Correct interpretation of experimental data is needed to build an adequate hypothesis about mechanism

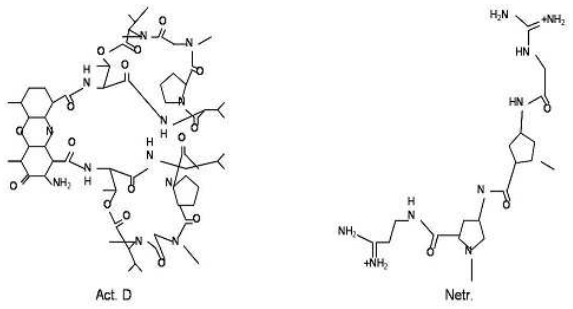

Figure 3: Structural formulae of anticancer antibiotics Actinomycin D and Netropsin (according to Neidle et al., 1987).

of linker histones/DNA interactions, as well as making clear the action of some drugs. We have formulated following assumptions as working hypotheses:

1. The preference of linker histones for binding to bent (kinked, curved) regions of DNA is considered to be proved (Yaneva et al, 1995; Yaneva et al., 1997). The conformational changes in DNA polynucleotide chain after Act. D treatment (some kind of DNA bending provoked by penetrating of peptide lactones of Act. D molecule into the minor groove) might provoke a facilitation of protein/DNA interactions.

2. The invasion of peptide lactones from the antibiotic molecule into DNA minor groove could arouse spatial extension of major groove and facilitation of linker histones binding to DNA.

3. Statistical analysis of antibiotic influence on linker histone/ DNA interaction

We can summarize the results of our experiments in the following Table 1, where the columns represent the possible three different effects of the antibiotic and the rows – different molar concentration of Act. D in the incubation mixture during the drug-competition assay.

That sort of data is naturally investigated by means of chi-square analyze. We used Matlab to complete the computations. The results we obtained lead to the following conclusions:

1. We can accept the hypothesis of independence of X and Y. This means the effect of Act. D does not depend on its concentration.

Xvs.Y	No effect (y_1)	Facilitation (y_2)	Inhibition (y_3)	Total
$10^{-3} = x_1$	10	7	5	22
$10^{-4} = x_2$	26	17	19	62
$10^{-5} = x_3$	42	16	14	72
$10^{-6} = x_4$	40	16	14	70
$10^{-7} = x_5$	38	11	22	71
$10^{-8} = x_6$	27	7	15	49
$10^{-9} = x_7$	4	4	3	11
Total	187	78	92	357

Table 1: Data on antibiotic influence.

2. The test of homogeneity for every two rows shows similarity of all of them, so there is a common distribution. The confidence intervals are:

p	MIN	AVG	MAX
p_0	0.4557	0.5238	0.5919
p_+	0.1622	0.2185	0.2748
p_-	0.1981	0.2577	0.3173

The results demonstrate a balanced effect of Act. D. on the linker histones/DNA interactions in case without preliminary treatment of DNA with antibiotic (so called "real competition assay"). The main table analyzed above represents data from the experiment with histone H1. We have created some additional tables with histone H5 (relative to H1) and with another antibiotic-Netropsin possessing another mode for binding to the double helix of DNA: it simply invades into the minor groove preferentially (if not only) in A-T reach stretches of the polynucleotide chain.

Comparative analysis of all the results infers some points of use:

1. For histone H5 there exists again common distribution with somewhat different parameters:

p	MIN	AVG	MAX
p_0	0.0243	0.1509	0.2776
p_+	0.5141	0.6792	0.8444
p_-	0.0370	0.1698	0.3027

2. For Netropsin we obtained dependency in χ^2 test. It means the behavior of Netropsin depends on its concentration.

3. We also have the case of preliminary incubation of that antibiotic with DNA. Here the resulting common distribution looks this way:

p	MIN	AVG	MAX
p_0	0.0663	0.1462	0.2260
p_+	0.5381	0.6462	0.7542
p_-	0.1160	0.2077	0.2993

This points out to strong facilitating effect of the antibiotic on the linker histones/DNA interactions in the case with preliminary treatment of DNA with the drug (competition assay).

4. Additional immunochemical assays and statistical inference.

Another type of experiments, based on ELISA (enzyme-linked immunosorbent assay) considers a hypothesis. Immunochemical methods are widely used in modern molecular biology. Antibodies (or immunoglobulins) are series of highly relative glycoproteins, produced in the organism as a result of the invasion of foreign protein or some polysacharides (Abbas et al., 2000). Sensibility of the reaction antigen – antibody is high enough to prove the presence, as well as the degree of association of an antigen with its complementary antibody. Nucleic acids (DNA, RNA) are not immunogens, but haptens, i.g. they are not able to challenge antibodies themselves, but complexed with proteins they do (Desai andMarion, 2000). A terrible disease, named Systemic Lupus Erythematosus exists, for which the presence of auto-antibodies against DNA and histone/DNA complexes is a reliable marker in differential diagnosis (Moens et al., 1995, Takuichi et al, 1995, Khalil at al., 1999). In spite of numerous investigations, the etiology of this disease is still unknown and usually the exact diagnosis has been pronounced during the advanced stage of the disease. As far as there are data showing the appearance of lupus-like syndrome and presence of anti-DNA antibodies after long-term treatment with anticancer antibiotics, scientists tend to accept the hypothesis that the reason for producing such pathogenic auto-antibodies could be conformational-modified DNAs, which in complex with circulating proteins, or even peptides, are identified as a foreign body in the organism (Desai and Marion, 2000). Our initial experiments aiming to prove such antibodies, treating with antibiotics rats with Guerin ascyte tumour induced are in process. As a referent a

polyclonal immune rabbit antiserum with antibodies against total histone/DNA complexes was produced (Zacharieva et al., 2004). Preparation of total histone includes linker histone H1 and core histones hH2A, hH2B, hH3, hH4 (Zlatanova and Yaneva, 1991a). The spectrum and the titer of the antibodies produced were firstly demonstrated with dot-immunobinding assay and then confirmed using ELISA (enzyme-linked immunosorbent assay). The results showed that the most part of antibodies in that polyclonal antiserum was against plasmid DNA compared with that to total histone and to complexes itself. We have obtained the following preliminary data – table 2.

pr.AG	DNA	Total h	t.h./DNA
1	0.224	0.189	0.182
2	0.175	0.168	0.194
3	0.115	0.060	0.078
4	0.212	0.193	0.166
5	0.233	0.193	0.211
6	0.178	0.162	0.189
7	0.183	0.127	0.176
8	0.168	0.138	0.133
9	0.078	0.042	0.050
10	0.209	0.163	0.181
11	0.238	0.184	0.157
12	0.199	0.171	0.156

Table 2: Here every row contains the indications (a value for unspecific binding with non-immune serum c=0.127 was subtracted from every single value of the ELISA-reader): for pure DNA antigen, pure histone H1 and histone/DNA complexes in 12 independent repetitions of the experiment.

Assumption is made that the first column (antigen pure DNA) differs from the other two. To confirm this, tests of Willcockson and of signs were performed and they showed that distribution of first column differs from the other two, which are similar (confidence level $\alpha = 0.05$). A drawing of empirical distribution functions illustrates distinction between case of pure DNA and the other two (Figure 4).

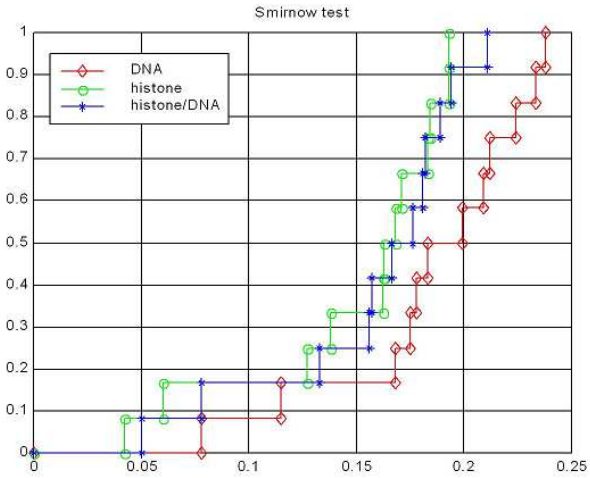

Figure 4: Empirical distribution functions.

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