

## EVIDENCE FOR THE CLOSE PROXIMITY OF HISTONES H1 AND H3 IN CHROMATIN OF INTACT NUCLEI

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### 1. Introduction

The most widely accepted model of chromatin structure is based on experiments with the chemical crosslinking of histones [1], nuclease digestion [2,3] and electron microscopy [4] of chromatin. In this model the elementary chromatin fibril consists of a linear array of repeating deoxyribonucleoprotein (DNP) subunits or nucleosomes. Each nucleosome contains two copies of each of the histones H2A, H2B, H3 and H4, histone H1 and ~200 base pairs of DNA. An octameric complex of the four histones (except histone H1) is the protein core of a nucleosome. This core is tightly associated with 140 base pairs of DNA to form a nucleosome 'core particle'. The tentative linear order of the attachment of histones to the core particle DNA has been reported [5]. As for histone H1, its precise location and biological function in chromatin still remain obscure. The analysis of DNP-fragments of chromatin generated by the action of nucleases indirectly supports the possibility of histone H1 binding to the internucleosomal 'spacer' DNA segments [6,7]. It is also believed that histone H1 interacts poorly if at all with the other histones but readily forms almost pure homopolymers in chromatin treated with bifunctional crosslinking reagents [8,9].

In the present work we also used reversible crosslinking of chromosomal proteins within intact cell nuclei to generate, isolate and then analyse the cross-linked polymers rich in histone H1. The study of such polymers by the methods of protein chemistry could obviously shed light on the spatial organization and location of histone H1 in chromatin.

### 2. Materials and methods

To obtain H1-containing histone polymers, thoroughly washed nuclei of calf thymus (5 mg/ml in DNA) were protected by the proteolytic inhibitor phenylmethanesulfonyl fluoride and then incubated for 1 h at 4°C with 10 mg/ml methyl-4-mercaptopurymidate hydrochloride (Pierce) (MMB) [8] in 10 mM Tris-HCl buffer, pH 7.9, made 0.25 M in sucrose, 5 mM in MgCl<sub>2</sub> and 0.03% (v/v) in  $\beta$ -mercaptoethanol. The reaction mixture was next dialysed against the same buffer but with mercaptoethanol and MMB omitted, and SH groups of the proteins attached to the MMB molecules were oxidized with 1% H<sub>2</sub>O<sub>2</sub> to produce S-S bridges crosslinking closely spaced histones. Unoxidized sulphhydryl groups were blocked by 2 mg/ml iodoacetamide. The suspension of nuclei was then dialysed overnight against 1 mM EDTA at 4°C, ultrasonicated at 44 kHz, and histones extracted with 0.2 M H<sub>2</sub>SO<sub>4</sub>. The extract was made 12% in trichloroacetic acid to precipitate histones, and the polymer fraction of the precipitate was isolated by gel filtration through a Sephadex G-200 (Pharmacia) column, equilibrated with 20 mM HCl. The fraction of interest moved with the void volume of the column.

Electrophoresis of histones in 15% acid-urea polyacrylamide gel was done according to [10]. Gels were stained with Coomassie brilliant blue R-250 (Serva). Histones were quantitated by scanning the gel tubes at 650 nm in a PMQ-II spectrophotometer (Zeiss) followed by integration of the scans. Since the mobility of the histones did not significantly change after modification with MMB, it was possible to identify the corresponding bands using purified histone

markers. The assignment was also supported by SDS-gel electrophoresis of the histones. In calculation of the molar ratios of different histone species we took into account the values of relative staining capacity for the histones in polyacrylamide gel obtained under similar conditions [11].

Antibodies specific to histone H1 were obtained by immunization of rabbits with the mixture of histone H1 (150  $\mu\text{g/ml}$ ) and the latter complexed with calf thymus linear fragmented DNA (1 mg/ml) according to the modified technique in [12]. The antibodies were additionally purified by passing them through a column of histone H1 molecules immobilized on BrCN-activated Sepharose (Pharmacia). The very high specificity of the recovered antibodies was substantiated by the method of immunoprecipitation in thin layers of agar (fig.1). Finally, antibodies against histone H1 were fixed on the carrier Affi-Gel 10 (Bio-Rad) and placed in a column.

### 3. Results and discussion

To detect histone constituents of the isolated polymer, the latter was incubated for 2 h at 37°C in

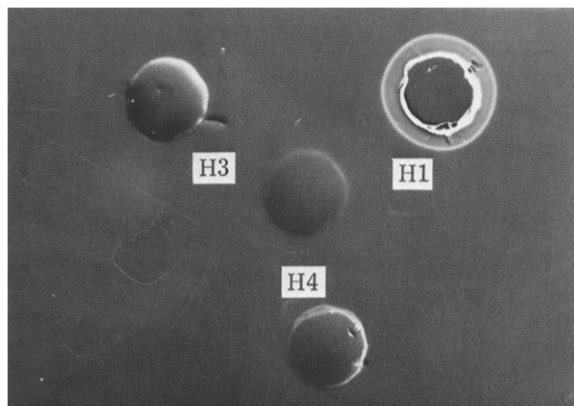


Fig.1. Immunoprecipitation test for the purity of anti-H1 antibodies. Purified H1, H3 and H4 histone fractions were placed in the holes and allowed to diffuse in a thin layer of agar (50 mM Na-phosphate buffer, pH 7.0). Unusual appearance of precipitation zones is due to sorption of histones on the acidic groups present in agar. The specific precipitation zone is only detectable around the H1-containing hole. Central hole contained antibodies.

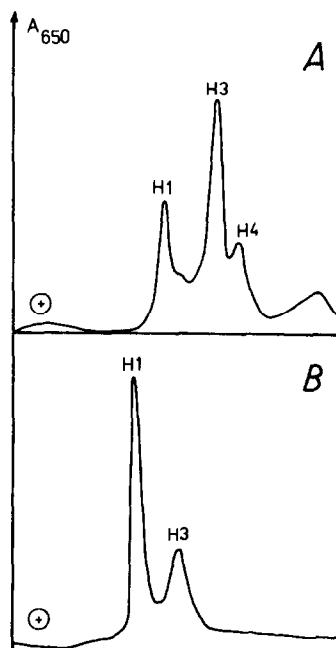


Fig.2. Densitometer traces of electrophoretically separated constituents of crosslinked histone heteropolymers reduced with  $\beta$ -mercaptoethanol.  $A_{650}$  in arbitrary units. (A) Heteropolymer (H1, H3, H4) from calf thymus; (B) heteropolymer (H1, H3) from rat liver.

10% (v/v)  $\beta$ -mercaptoethanol which resulted in complete reduction of disulfide bridges with the concomitant splitting of the original high molecular weight compound into individual histone fractions. As judged from the densitometer trace of electrophoretically separated reduction products depicted in fig.2A, the polymer in question consists of the histones H1, H3 and H4 in molar ratios of 1:2:1.3, respectively.

Unreduced polymer fraction does not enter 15% polyacrylamide gels indicating min. mol. wt 100 000 with a corresponding degree of 'protein polymerization' of no less than 5.

To prove that the polymer obtained really represents a heteropolymer (H1, H3, H4) of crosslinked histones and not a physical mixture of certain unseparated polymers (e.g., homopolymers of histones H1, H3 and H4), an immunochemical test was carried out. The polymer (H1, H3, H4) in 50 mM Na-phosphate buffer, pH 7.0, 0.25 M NaCl, was passed through a column filled with immobilized antibodies specific

to histone H1. As a result, the protein was completely retained in the column. Inasmuch as histone H1 is well known to have no tendency to associate with any other histones in solution, this finding indicates the participation of covalent bonds in attachment of histones H1, H3 and H4 to the polymer chain.

On the other hand, the possibility of simultaneous independent formation of poly (H1) and high molecular weight polymers composed of histones H3 and/or H4 seems rather unlikely [8,9]. The conclusion from the immunochemical test is in accordance with these results.

The data reported above are inadequate to unambiguously establish the pattern of histone-histone crosslinking in the polymer. But we know the proportion between the constituent histones H1, H3 and H4 in the heteropolymer, 1:2:1.3. Moreover, using a lower excess of MMB in rat liver nuclei we have succeeded in isolating and characterizing a related copolymer (H1, H3) of histones H1 and H3 in the molar ratio 3:1, respectively. The copolymer contains a considerably smaller fraction of histone H3 and completely lacks histone H4 (fig.2B). Finally, the procedures of histone crosslinking in chromatin similar to those employed here have been shown to rather effectively induce formation of dimers and some higher oligomers of histones H3 and H4 [8,9].

A comparison of the evidence just cited leads to the following tentative conclusions:

1. The molecules of histone H1 as the constituents of chromatin can be crosslinked with a middle size bifunctional reagent (the length of MMB is about 5.5 Å) to a homopolymer chain without the interspersed of other histones. In other words, a structural backbone of the heteropolymer (H1, H3, H4) is an H1-homopolymer bearing the histones H3 and H4 apparently attached as side chains.
2. Under sufficiently favourable conditions of crosslinking the stoichiometry of histones H1 and H3 in the heteropolymer produced (H1, H3, H4) is the same as in a nucleosome, i.e. two H3 molecules per one molecule of histone H1.
3. Histone H4 combines with the heteropolymer (H1, H3, H4) as a part of a preformed heteroassociate (e.g., dimer) with histone H3 rather than directly.

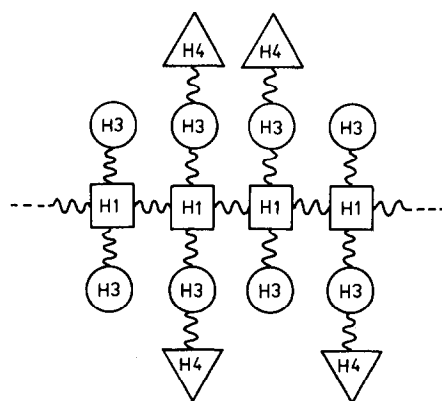


Fig.3. Putative pattern of the MMB-crosslinked heteropolymer (H1, H3, H4) structure. Wavy lines, MMB; geometrical figures, histones.

It follows that the treatment of chromatin in intact nuclei with the reversible bifunctional crosslinking reagent MMB results under appropriate conditions in the homopolymerization of histone H1 molecules followed by the covalent attachment of histones H3 and H4. The practically stoichiometric (in terms of protein content of a nucleosome) binding of histone H3 to the H1 polymer in calf thymus nuclei suggests that in virtually intact chromatin at least part of the H1 polypeptide chain is localized in close proximity to a nucleosomal histone core and even probably occupies particular segments of the 'core particle' DNA.

There is some reason to assume that the fragment of histone H1 interacting with the 'core particle' DNA is the N-terminal half, roughly organized as a cationic structureless segment of amino acid residues 1-40 and a structured apolar segment 41-120 [13]. In this case the C-terminal half of the H1 could be bound to any other part of a nucleosome including also the internucleosomal 'spacer' DNA. The highly conservative primary structure of the globular part of histone H1 is potentially capable of specific binding to the likewise conservative DNP 'core particle' with the contribution of hydrophobic and/or H-bonds. This kind of interaction is able to govern the orientation of histone H1 molecules with respect to a nucleosomal chain, the stoichiometry of the H1 binding to DNA (1 H1 molecule/nucleosome), the distribution of histone H1 subfractions, and other important features of the chromatin structure.

The work of establishing the crosslinked parts of histones H1 and H3 in the heteropolymer (H1, H3, H4) in terms of different peptides is now in progress in our laboratory.

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