The accessibility of histone H5 in chromatin was examined with monoclonal antibodies recognizing several epitopes of the globular region (GH5) of the histone (Rozalski, M., Lafleur, L., and Ruiz-Carrillo, A. (1985) J. Biol. Chem. 260, 14379-14385). The stoichiometry of the chromatin-antibody complexes indicated that while 0-86% of the H5 molecules were able to react, depending on the particular epitope, the extent of antibody binding to relaxed chromatin (in 5 mM KCl) and condensed chromatin (in 100 mM KCl or 0.35 mM MgCl₂) was virtually identical. This indicates that the topography of H5 does not change during the conformational transition of chromatin. The data suggest that H5 is not completely internalized in the 30-nm fiber or that the fiber is flexible enough to allow full exposure of the GH5 epitopes. Several control experiments, including monoclonal antibody binding, sedimentation analysis, DNase II digestion, and glutaraldehyde cross-linking, showed that epitope accessibility is not due to H5 exchange or to perturbation of the chromatin fiber. The accessibility of GH5 suggests ways in which inactive chromatin may be unfolded in vivo.

Transcriptionally inactive chromatin from the intermitotic cells of higher eukaryotes appears as fibers of approximately 30-nm diameter (1). In vivo, this structure results from condensation of a relaxed poly(nucleosome) chain by a variety of actions (see for instance Ref. 2) and requires the presence of the linker histone H1 or variants of it like H5 (3-5). The globular region of the linker histones has been shown to interact with the nucleosome at the entry and exit sites of DNA (6, 7), probably sealing the particle and correctly positioning the C termini (6, 8). On the other hand, the topography of H1 or H5 on the 30-nm chromatin fiber is not known. Assuming that the nucleosome binding site(s) is preserved in the higher order structure, various models predict different locations for these histones (1). Since the 30-nm chromatin fiber most likely constitutes a barrier to polymerases, the cell must possess a mechanism to unravel it. The linker histones could, if accessible, act as a key element in this process because of their involvement in the organization (3, 4) and in the maintenance (5, 9) of the higher order structure. Therefore, the location of the linker histones in condensed chromatin is of considerable interest from structural and functional viewpoints.

We have examined the accessibility of different epitopes of the globular region of H5 (GH5) to nondegradative probes using well characterized (10) monoclonal antibodies (mAb). H5 was selected because it is the major linker histone in chicken erythrocyte chromatin (i.e. 75% H5, 25% H1; Ref. 11), and previous work has shown that there is a molecule of H1 or H5 per nucleosome (12). In addition, mature erythrocyte chromatin has a simple composition, it is very stable in vitro, and it is mostly transcriptionally inactive (Ref. 11, and references therein).

**MATERIALS AND METHODS**

**Preparation of Soluble Chromatin**—Hen erythrocyte nuclei were obtained in buffer A containing 0.5% Nonidet P-40. Calf thymus nuclei were obtained in buffer A. Nuclear pellets were washed in buffer A and digested with micrococcal nuclease (150 units/ml, 5 mg DNA/ml) in the same buffer, containing 2 mM MgCl₂ and 1 mM CaCl₂, for 35 min (erythrocytes) or 25 min (lymphocytes) at 4°C. The reaction was stopped by the addition of EGTA to 3 mM.

The suspension of digested nuclei was dialyzed for 16 h at 4°C (5) against B5 or B6. Size-fractionated chromatin (average length of 120-130 nucleosomes) was prepared by sedimentation of the solubilized chromatin in 5-20% linear sucrose gradients and dialyzed against B5. Chromatin was condensed by a continuous increase in the concentration of KCl (3.96 mM/h) or MgCl₂ (0.015 mM/h) by dialysis at 4°C. The starting buffer was B6 for Blm-chromatin and B5 for BMg-chromatin. Chromatin was used between 10 and 24 h after its preparation, and its integrity (histones and DNA) was verified prior to and after the experiments. The hydrodynamic behavior of B5-m-chromatin was equivalent to that of chromatin solubilized at physiological ionic strength (5).

**Cross-linking of Chromatin**—Chromatin (100 µg/ml) in B6 or BMg was reacted in the dark with freshly deionized glutaraldehyde (7-9 µS) for 15 h at 4°C. The reaction was quenched with 50 mM ethanolamine-HCl (pH 7.3) at 0°C for 30 min and extensive dialysis against the desired buffers. Digestion of soluble chromatin, or chromatin-antibody complexes, with micrococcal nuclease or PMSF-treated DNase II was performed as indicated in the text. Digested chromatin was subsequently treated with 0.14 M HCl (pH 2.3) for 2.5 h at 60°C or 0.14 M NaOH (pH 11.7) for 0.5 h at 37°C. After the treatment, the solution was neutralized (final 18 mM NaCl, pH 7.2), and chromatin was absorbed on microtiter wells.

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The Globular Region of Histone H5 Is Equally Accessible to Antibodies in Relaxed and Condensed Chromatin*

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* The abbreviations used are: mAb, monoclonal antibody; EGTA, ethylenediamined(tetraethylenenitrilo)tetracetic acid; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; buffer A, 0.25 mM sucrose, 3 mM MgCl₂, 0.4 mM PMSF, 20 mM Hepes-NaOH (pH 7.2); buffer B6, 0.05 mM EGTA, 0.4 mM PMSF, 5 mM Hepes (pH 7.3); buffer B5, 5 mM KCl, 0.05 mM EGTA, 0.1 mM EDTA, 0.4 mM PMSF, 5 mM Hepes-NaOH (pH 7.3); buffer BMg, 100 mM KCl, 0.05 mM EGTA, 0.1 mM EDTA, 0.4 mM PMSF, 5 mM Hepes-NaOH (pH 7.3); buffer BMg, 35 mM MgCl₂, 0.05 mM EGTA, 0.4 mM PMSF, 5 mM Hepes-NaOH (pH 7.3); GH5, globular region of H5. Tryptic peptide encompassing residues 22-100.
Antibody Binding to Chromatin—Purified anti-GH5 mAb (10) were free of contaminating proteolytic and nucleolytic activities. mAbs were labeled with [3H]CHCl3 (Du Pont-New England Nuclear, specific activity 78 Ci/mol) by reductive methylation (13) or with Bolton-Hunter reagent (ICN Radiochemicals, specific activity 2453 Ci/mmole). The specific radioactivity of the antibodies varied between 1.52 × 10⁶ and 2.75 × 10⁶ cpm/μg. Fab' fragments were obtained by treatment of complexes with 8 mM cysteine. Free sulfhydryl groups were blocked with 50 mM N-ethylmaleimide at 37 °C for 30 min. Fab' fragments were purified by dialysis or protein A-Sepharose chromatography.

Standard reactions (1 μg of chromatin-DNA/75 μl reaction) were carried out in solution in the indicated buffers containing 0.1 mg/ml bovine serum albumin and 0.05% Nonidet P-40 at 22-24 °C for 2 h. Nonidet P-40 had no influence on the extent of the reaction but helped to produce more compact pellets. Chromatin-antibody complexes were precipitated by addition of 25 μl of 40 mM MgCl₂ in buffer B₅. After centrifugation, the pellets were quickly rinsed with buffer B₀ containing 10 mM MgCl₂ and 0.05% Nonidet P-40. MgCl₂ effectively precipitated chromatin as judged from analysis of the pellets and supernatants. All reactions were carried out in duplicate, and the values given in the text are the average of two independent experiments. The amount of antibody bound to chromatin was determined by densitometry (Chromoscan 3, Joyce-Loebl) of the histones after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of duplicated chromatin samples. Purified H₅ (the concentration of which was determined by amino acid analysis), run in the same gel, was used as standard. The extent of antibody binding to chromatin is expressed as molar ratios, from the known specific radioactivity of the antibodies and the amount of H₅ in chromatin, taking 146 and 21.6 kDa as the molecular masses of IgG and H₅, respectively. DNA concentration was determined by fluorimetry.

Solid phase radioimmunoassays were performed essentially as described (10). H₅/DNA complexes (0.2/1.0, w/w) were adsorbed on microtiter wells in B₅ or B₅₀. All coated wells were rinsed three times with B₀₀ prior to incubation with the antibodies, to remove loosely bound complexes. Glutaraldehyde cross-linked chromatin was absorbed and reacted in B₅ or B₅₀. The degree of absorption of chromatin in B₅ or B₅₀ was comparable.

RESULTS

Reactivity of Chromatin Fibers Condensed with KCl—The topography of H₅ in chromatin was examined by determining the extent of binding of several mAbs specific for GH5 (10). The accessibility of the different epitopes should depend on the degree of H₅ exposure, provided that the structure of chromatin is not destroyed by the antibodies and that possible H₅ rearrangement is controlled for. If these conditions are met, GH5 accessibility must be maximal in the relaxed conformation, whereas it should depend on the degree of H₅ internalization in the higher order structure of the chromatin fiber. Thus, the extent of mAb binding in the two conformations of chromatin can be related to changes in GH5 exposure.

In the first series of experiments, size-fractionated chromatin was relaxed in 5 mM KC1 (B₅-chromatin) or condensed in 100 mM KC1 (B₅₀-chromatin) (see "Materials and Methods"). Equal amounts of chromatin were incubated separately with increasing amounts of several radiolabeled mAbs for different periods of time. The extent of mAb binding was determined after precipitation of the chromatin-antibody complexes with 10 mM MgCl₂, and it is expressed as molar IgG/H₅ ratios. In general, binding of the mAb to chromatin reaches equilibrium within 1 h of incubation (no change in the stoichiometry of the chromatin-mAb complex was observed after incubation periods of up to 70 h). However, one of the mAbs, 4C₃, needed incubation periods of up to 48 h in B₅₀ before reaching equilibrium, unless a vast excess was used. This behavior is due to a lower affinity of 4C₃ for H₅ at the higher ionic strength and not to the inaccessibility of GH5 (see below, Fig. 4C and Table I).

The results obtained with 5C₃ and 3G₈, recognizing different epitopes of GH5, are shown in Fig. 1, A and C. Clearly, binding of these mAbs was essentially independent of the state of chromatin condensation, and the plateau values are comparable for a given mAb, although each antibody saturates chromatin to a different level (i.e., about 60 and 30% of the H₅ molecules were recognized by 5C₃ and 3G₈, respectively; see Table I). Similar results were obtained at intermediate ionic strength (40 mM KCl) or when chromatin was extracted at 100 mM KCl (not shown). Control experiments showed that no precipitation of the chromatin-mAb complex occurred spontaneously and that no detectable amount of antibody was precipitated by 10 mM MgCl₂ when the reactions were carried out in the absence of chromatin. Binding of the antibodies depended on the presence of H₅, since no complex was observed when the reactions were carried out with calf thymus chromatin (not shown; see also Ref. 10, Figs. 2, 3, and 7). Furthermore, polyacrylamide gel electrophoresis of the precipitate and supernatant fractions after MgCl₂ precipitation of the chromatin-antibody complexes showed no evidence for any detectable release of H₅ into the supernatants. Results of chromatin titration on solid phase, not involving a MgCl₂ precipitation step, were in complete agreement with those obtained in solution (see Fig. 7).

![Fig. 1](image-url) Binding of ¹²⁵I-labeled anti-GH5 mAb to chromatin (A and C) and H₅/DNA complexes (B and D). Chromatin (solid phase) and H₅/DNA complexes (solid phase) were incubated with increasing amounts of mAb in B₅ (C) and B₅₀ (D). The extent of mAb binding was determined from the radioactivity bound to the solid phase (B and D), or to the chromatin-mAb complexes after precipitation with 10 mM MgCl₂. The amount of mAb bound to chromatin (A and C) is expressed as molar IgG/H₅ ratios (see "Materials and Methods" for further details).

![Fig. 2](image-url) Inaccessibility of the GH5 epitopes presumably located at the entry and exit site of DNA in the chromatosome. Binding of ¹²⁵I-SH₁₀ to soluble chromatin (A) or plastic-bound H₅/DNA complexes (B). A, 2 μg of chromatin were incubated with 5 μg of the mAb for the indicated periods of time. In B, the incubation was for 2 h. B₅₀ (○), B₅ (□), and B₀ (△). M.R., molar ratios (see legend to Fig. 1 for further details).
To interpret the data in terms of GH5 accessibility, we first verified that the results were not affected by H5 exchange, by changes in the relative affinity of the antibodies at the two ionic strengths, or by perturbations in the structure of chromatin caused by binding of the mAb. The possibility that the results obtained at B100 were influenced by H5 exchange (15) was examined with 5H10, a mAb that recognizes a repeated GH5 epitope (10). 5H10 reacts at 100–150 mM KC1 with the protein in solution (10) or in DNA complexes (Fig. 2B), but not in chromatin (Fig. 2A) or whole nuclei (10). The trivial explanation that the lack of reactivity with chromatin (in B6 or B100, Fig. 2A) is due to partial exchange of the H5-5H10 complex from chromatin was dismissed by analysis (polyacrylamide gel electrophoresis) of the MgCl2-precipitated chromatin (not shown). Hence, the most reasonable interpretation of the data is that the GH5 epitope is not accessible to the antibody. This is supported by the reactivity of 5H10 toward chromatin in B6 (Fig. 2A). At 0 mM KC1 the nucleosome unfolds because of the reduced electrostatic shielding of the DNA-phosphate backbone (see, for instance, Refs. 16 and 17). The unfolding of the structure presumably displaces H5 from its normal binding site, thus exposing the epitope otherwise inaccessible. From the above observations, it follows that if H5 were to exchange in B100 during the reaction, then 5H10 should be able to bind to the epitope. Therefore, we suggest that the lack of reactivity of 5H10 with chromatin in B100 (or B6) can be taken as an indication that there is either no H5 rearrangement or that the kinetics of exchange is faster than the reaction with the antibodies. In either case, the results obtained with the other mAbs are unlikely to be affected by this possible phenomenon. A similar conclusion can be reached from antibody binding experiments to chromatin condensed in MgCl2 (see below).

Although the extent of mAb binding to chromatin in B6 and B100 was comparable, the GH5 epitopes appeared to react slightly better with 5C3 in B100 than in B6, whereas at the lower mAb concentrations the opposite was true for 3G8 (Fig. 1, A and C). This behavior suggested that the variations were due to changes in the affinity of the mAb at the two ionic strengths, rather than to the conformation of chromatin. That this is the case is shown in Fig. 1, B and D, where the effect of salt was examined by solid phase radioimmunoassays using H5-DNA complexes. Clearly, binding of the mAb to GH5 in the DNA complex or in chromatin followed a similar trend, suggesting changes in antibody affinity as a function of the salt concentration.

The possibility that the results obtained with B100-chromatin (Fig. 1, A and C) were due to structural perturbations induced by the mAb was also examined. If binding of mAb were to disturb the higher order structure, one would predict considerable unraveling of the fiber since the IgG/H5 ratios of condensed and relaxed chromatin are comparable. Moreover, displacement of only 10–15% of the linker histones has a profound effect on the hydrodynamic properties of chromatin (5). The sedimentation profile of chromatin reacted with 4C3 in B100 (2 IgG molecules bound/10 nucleosomes) indicated no appreciable changes in the structure of chromatin since its average S value was not noticeably affected by the antibody. This is expected if an increase in mass is compensated by an increase in the frictional coefficient (i.e., assuming a rod-like structure for B100-chromatin, S should increase by a factor of 1.06; Ref. 18). The experiment in Fig. 3 also demonstrated that the amount of 4C3 bound to chromatin was only slightly lower (10–20%) than that determined by MgCl2 precipitation. The small difference is probably explained by antibody dissociation due to dilution of the complex in the gradient.

From the above data, we conclude that the extent of mAb binding to chromatin does reflect the accessibility of GH5.

Reactivity of Chromatin Fibers Condensed with MgCl2—The above results were confirmed by the use of Mg2+ as an alternative counterion for chromatin condensation. The much lower salt concentration of MgCl2 than the KCl needed to achieve an equivalent degree of condensation should also minimize any change in the affinity of the antibodies, as well as decrease any randomization of histone H5. The structural effect of MgCl2 on chromatin was determined by sedimentation analysis. It was found that 0.30–0.35 mM MgCl2 in B6 produced the highest degree of condensation compatible with solubility (i.e., the S value of chromatin in B100 and B6 was indistinguishable, not shown), in agreement with a previous report (19). Increasing the MgCl2 concentration further (i.e., to 0.40 mM and higher) did not result in a shift in S but in a progressively lower recovery of chromatin due to precipitation. Hence, chromatin was routinely condensed in B6 containing 0.35 mM MgCl2 (B6, see “Materials and Methods”).

Binding of 4C3 and 5C3 IgG to chromatin in B6 and B5 was again indicated that the reactivity of GH5 is independent of the state of chromatin condensation (Fig. 4, A and C). In fact, the titration curves in the two buffers are nearly superimposable, and the saturation level of B5-4C3-chromatin with 5C3 was comparable to that of B100-chromatin (cf. Figs. 1A and 4A, Table I). As opposed to the slow kinetics of reaction in B100 (see above), 4C3 reached binding equilibrium within 1 h in B6, confirming that the affinity of the antibody, rather than the accessibility of GH5, was affected by the ionic strength of the medium. The saturation values of 4C3 and 3G8 (Table I) were also similar, as expected from the high degree of overlap of the two epitopes (10).

Sedimentation analysis of chromatin-4C3 complexes in B5 was

![Fig. 3. Structural integrity of chromatin after mAb binding. Sedimentation profiles of chromatin in B100 after centrifugation through sucrose gradients. Equal amounts of chromatin were mock-incubated (a) or incubated (b) with 1H-labeled 4C3 before centrifugation. 1H-radioactivity is represented by the open columns. The A450 of the top fractions (b) is due to the large excess of free mAb and the bovine serum albumin present in the reaction. The broken line indicates the base line of profile (a). The peak of the distribution of chromatin relaxed in B6, run in a parallel gradient, is indicated by the arrow.](image-url)
The extent of mAb binding was determined as indicated in the legend to Fig. 1. B6-chromatin gave results identical to those of B6-chromatin and BM,-chromatin was digested with DNase II (20) of B5-chromatin (Fig. 5, lane a and c), although it slowed the reaction. The latter effect is probably due to steric hindrance of the nuclease cutting sites by the bulky mAb. Digestion in B5 of chromatin cross-linked in BMg (Fig. 6c, lane c) demonstrated that, indeed, the DNA fragment patterns reflect chromatin organization rather than an intrinsic change in the mode of action of DNase II in BMg. These results indicate that the structure of chromatin condensed by Mg2+ is not perturbed by the mAb, and that the accessibility of GH5 in relaxed and condensed chromatin is independent of the counterion used to induce condensation.

The saturation levels of the different mAb may reflect steric hindrance or the divergent character of the IgG. To distinguish between these, we conducted binding experiments with chromatin and Fab' fragments. The results with 5C3 Fab' showed that the extent of the reaction was comparable to that obtained with the complete IgG (Fig. 4b, Table I). 4F2 Fab', which shows partial interference with 5C3 and 3G8 (or 4C3) (10), gave intermediate levels of saturation (Fig. 4d, Table I). Although similar experiments with 3G8 and 4C3 were not possible due to the low affinity of the Fab's, the results obtained with 5C3 and 4F2 Fab' indicate that the extent of mAb binding is mostly independent of the valence and size of the mAb.

Reactivity of Cross-linked Chromatin Fibers—In an attempt to distinguish whether the reactivity of GH5 in chromatin reflects its exposure or the flexibility of the fiber (local opening), chromatin was fixed with minimal amounts of glutaraldehyde, such that the higher order structure would be "locked" at a minimum cost in epitope loss by chemical modification. Fig. 6a shows the products of histone cross-linking of chromatin in B5 or BMg. As expected, the main products of the reaction were homo(co)polymers of H1 and H5 (21), although the core histones were also cross-linked among themselves and/or to H1/H5 oligomers. As previously reported (5), the rate of histone cross-linking is higher in condensed than in relaxed chromatin due to the closer propinquity of the histones (Table II). Judging from the hydrodynamic behavior of the DNA fragment patterns of chromatin in B5 or BMg (Fig. 5, lanes a and c), both the IgG and Fab' fragments appear to be immobilized in the condensates.

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with the indicated amounts of glutaraldehyde. After dialysis against BS, chromatin was adsorbed on microtiter wells without further treatment. The drop in reactivity of BxM-chromatin is 2.2-2.4 higher than of BxS-chromatin.

Chromatin (0.015% glutaraldehyde) digested with micrococcal nuclease before (lane a), with HCl (lane b), and after HCl treatment (lane c; lane a, HinfI fragments of pBR322 DNA, 1-3 (in base pairs), 1631, 511, and 221, respectively. B), analysis of BxM-chromatin (0.015% glutaraldehyde) digested with micrococcal nuclease before (lane b), and after HCl treatment (lane c; lane a, HinfI fragments of pBR322 DNA, 1-3 (in base pairs), 1631, 511, and 221, respectively. B), analysis of BxM-chromatin (0.015% glutaraldehyde) digested with micrococcal nuclease before (lane b), and after HCl treatment (lane c; lane a, HinfI fragments of pBR322 DNA, 1-3 (in base pairs), 1631, 511, and 221, respectively.

Three aspects of the data are worth pointing out. First, there is a decrease in mAb binding to chromatin with the increase in the cross-linker concentration. Second, at 0.009% glutaraldehyde about one-half of the GH5 epitopes of BxM-chromatin (compared to BxS-chromatin) are still reactive. Third, the drop in reactivity of BxM-chromatin is 2.2-2.4 higher than of BxS-chromatin.

The decreased reactivity of the GH5 epitope in BxM-chromatin with respect to that of BxS-chromatin cannot be solely explained in terms of chemical modification since the time of the reactions, and the chromatin and the cross-linker concentrations were identical. The relative higher inaccessibility of GH5 in BxM-chromatin may reflect the location of the molecule, deep within the structure of chromatin. Alternatively, this inaccessibility may be due to the stabilization of molecular contacts that mask the epitope, contacts that would occur only transiently in the uncross-linked state. The latter alternative does not necessarily require GH5 to be buried inside the structure of the chromatin. To distinguish between the two possibilities, we examined the binding of 5C3 to Bx, BxM, BxS, and BxM-chromatin after limit digestion with micrococcal nuclease and incubation in NaOH or HCl. The results (Fig. 7A) demonstrated that neither treatment significantly affected the extent of 5C3 binding. Since the duplex DNA backbone was effectively destroyed by the treatments (e.g., Fig. 7B), and with it the higher order structure of the chromatin, the results strongly support the view that the apparent higher inaccessibility of GH5 in BxM-chromatin is due to a permanent masking of the epitope. Concerning which of the histones might be involved in this modification, Table II shows that there is a striking parallelism between the rate of H3 cross-linking and the loss of GH5 reactivity. Since no cross-linked H5 dimers were detected (Fig. 6A; H3 dimers would migrate between the slowest H1 variants), it follows that H3 was cross-linked to H1/H5. From this we suggest that the lower reactivity of GH5 in BxM-chromatin is primarily caused by occlusion of the 5C3 epitope, most likely by cross-linking to H3, and not necessarily by the internalization of GH5 in the structure of the chromatin fiber. However, just these results cannot rule out an internal location of GH5 at the time the H5-H3 cross-links were made.

DISCUSSION

Our work with uncross-linked chromatin has indicated that the accessibility of GH5 to mAb (10) is not affected by the conformational changes accompanying chromatin condensation in 120-130 long nucleosome oligomers. This conclusion is based on the titration of several GH5 epitopes that react to different levels (Table I). The results were independent of the cation used to induce the higher order structure of chromatin (K+ or Mg2+) and of the method of analysis (solution or solid phase). A number of controls have ruled out that the reactivity of GH5 is due to perturbation of the chromatin structure, H5 exchange, or H5 displacement by the mAb. Additional experiments have also demonstrated that the extent of mAb binding to shorter chromatin fibers (average length of 60 nucleosomes) is not dependent on the length of the fibers used (not shown). This, and the high proportion of H5 molecules recognized by 5C3 and 4F2, argues against the possibility that our results are significantly affected by small regions of unfolded chromatin, which may exist at the ends of a finite fiber.

The constant accessibility of the GH5 epitopes during the condensation of chromatin strongly suggests that the framework of the higher order structure already exists in the relaxed conformation. This is in accordance with the view that condensed chromatin results from the progressive folding of a pre-organized structure (27, 28). The same argument suggests that the topography of GH5 in the chromatome (6) and in the 30-nm chromatin fiber is very likely the same. The reactivity of the epitopes was independent on the size of the antibodies (IgG or Fab'). Although Fab', due to its smaller size, could conceivably diffuse through the fiber, IgG cannot. This argues that GH5 is not located deep inside of the fiber or that the chromatin is flexible enough to allow the probes to reach the interior of the structure.

The experiments with fixed chromatin were aimed at distinguishing between exposure of GH5 and flexibility of the 30-nm fiber. By carefully controlling the cross-linking reaction, conditions were found (i.e., 0.009% glutaraldehyde) that prevented unraveling of the chromatin. Under these conditions, the reactivity of GH5 in BxM-chromatin was reduced to about half of that in BxS-chromatin. Since increasing the extent of

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Table II

<table>
<thead>
<tr>
<th>Glutaraldehyde (%)</th>
<th>5C³ binding</th>
<th>Cross-linked histones</th>
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<td>0.009-Bs</td>
<td>75</td>
<td>H3 60 75 95</td>
</tr>
<tr>
<td>0.012-Bs</td>
<td>65</td>
<td>H2B 42 61 85</td>
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<td>H4 51 51 46</td>
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</tr>
<tr>
<td>0.015-BxS</td>
<td>14</td>
<td>9 9</td>
</tr>
</tbody>
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* Expressed as percentage of that in noncross-linked chromatin.
* Expressed as percentage of core histones remaining as monomers.

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3 A. Ruiz-Carrillo, unpublished observations.
cross-linking did not result in appreciable changes of the hydrodynamic properties of the fiber, we argue that the results are representative of the bulk chromatin, rather than of a particularly compact conformation. Hence, unless changes in chromatin flexibility do not affect its sedimentation behavior, the results suggest that the 5C3 epitope is not inside the fiber. We have also presented evidence indicating that the gradual loss of 5C3 reactivity with the increase in glutaraldehyde concentration is due to the progressive occlusion of the epitope, most likely through GH5-H3 cross-linking (Fig. 6A, and Table II), contacts that presumably reflect transient interactions between these two histones in the higher order structure of chromatin. Due to this complication, the cross-linking data do not permit to completely discriminate between GH5 exposure and fiber flexibility. The higher masking of the GH5 epitope in B
\text{X}_{64}\text{-chromatin (Table II) suggests, nevertheless, that regions of H3 are as external as GH5, in agreement with protease digestion studies (5, 25, 26). In any event, if 5C3 reacts with H5 through cavities between adjacent nucleosomes the crevices should be at least 5 x 5 nm wide and probably not be deeper than 5-6 nm, given the dimensions of the Fab. The accessibility of H1 or H5 in chromatin has been previously examined using polyclonal antibodies, and the results have been varied (22, 23). The earlier work, however, was qualitative and changes in the affinity of the antibodies at the different ionic strengths were not examined. We have shown that the affinity of 4C3, used in our work, was very sensitive to changes in ionic strength. Binding experiments using anti-H5 polyclonal antibodies (12) showed that the affinity of 40-50% of them were seriously affected at 100 mM KCl. Due to the complexity of the polyclonal probes, buffer-dependent changes in affinity could easily be misinterpreted in terms of variability in histone accessibility.

After this work was completed, another report has appeared in which the binding of polyclonal antibodies to GH5 was examined with heavily cross-linked (0.1% glutaraldehyde) chromatin (24). The results showed a decrease in GH5 reactivity with the ionic strength of the cross-linking medium. This was interpreted in terms of inaccessibility of H5, and lead the authors to propose that GH5 is internally located in the 30-nm chromatin fiber. We have shown the limited usefulness of glutaraldehyde cross-linking to address the question of GH5 accessibility in chromatin because of the permanent loss of the GH5 epitope (Fig. 7), apparently apparent by cross-linking with H3. Since H3 is digested with trypsin at a similar rate that the C-terminal regions of H1 or H5 (5, 25, 26), our findings may also explain why Dimitrov et al. (24) measured a higher GH5 reactivity in trypsin-treated chromatin, regardless of the ionic strength, than in chromatin relaxed at 1 mM salt.

Given the prominent role of the linker histones in the organization (3-5) and maintenance (5, 9) of the higher order structure of chromatin, the in vitro accessibility of GH5 suggests ways whereby chromatin can be activated in vivo. Thus, enzymes that modify the affinity of the linker histones for DNA could interact with them and alter their binding properties, thereby entailing the opening of the fiber (and perhaps of the nucleosome, too). This will endow chromatin with the necessary plasticity to respond to metabolic needs (e.g. replication).

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