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Iodination of nucleosomes at low ionic strength: conformational changes in H4 and stabilization by H1

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ABSTRACT

Radioactive iodine has been used to probe the relative reactivities of nucleosomal H4 tyrosine residues under various conditions of subphysiological ionic strength. We observe that tyrosine 72 of H4, which is not reactive over the range 20-150 mM NaCl, becomes the predominant site of iodination within H4 when nucleosomes are subjected to conditions of very low ionic strength. Conversely, the other H4 tyrosine residues, which are reactive within nucleosomes in solutions of moderate ionic strength (20-150 mM NaCl), become nonreactive when the ionic strength is reduced. This "flip-flop" in the H4 iodination pattern is the manifestation of a reversible nucleosomal conformational change. A method is presented which enables the conformational status of H4 in nucleosomes to be determined by simply electrophoresing the histones on a Triton gel after probing nucleosomes with labeled iodine. Using this technique, we demonstrate that the presence of H1 on one side of the nucleosome stabilizes a histone core domain on the other side so that all four tyrosines of H4 are maintained in their physiological ionic strength conformation even under conditions of no added salt.

INTRODUCTION

The subunit structure of chromatin is by now a familiar story.[1] The DNA is known to be organized at the primary level into a tandem array of nucleosomal units of somewhat variable lengths. The variability is confined to the so-called "linker" DNA which is particularly sensitive to micrococcal nuclease. Brief digestion with this nuclease reveals a fundamental unit (the "chromatosome") which contains 168 base pairs of DNA[2,3] wrapped in two turns[1,3,4] about a histone core comprised of two each of the histones H2A, H2B, H3, and H4 with an additional histone, H1, located on the periphery. When H1 is removed, an additional 11 base pairs at each end show enhanced sensitivity to micrococcal nuclease and are easily degraded to yield the core particle (146 base pairs). This particle has been subjected to extensive structural analysis.[1]

The probability that even transcriptionally active chromatin is packaged into nucleosomes[5,6] has led to much speculation as to how RNA poly-

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merase might be able to negotiate these structures. To approach this issue, we have studied various aspects of the ability of nucleosomes to change conformation.[8,9,10] Among the nucleosomal conformational changes which we have investigated are those induced by the electrostatic stress of low ionic strength.[8,9] In one study we used hydrodynamic techniques to elucidate the ways in which two different structural features modulate the ability of nucleosomes to "unfold" at low ionic strengths.[8] We showed that the length of the component DNA is relatively unimportant above chromatosome size but that nucleosomes containing shorter pieces of DNA (i.e., core particles) may exhibit a somewhat truncated response. We also demonstrated that the presence of H1 stabilizes nucleosomes and prevents this unfolding from taking place. However, a limited amount of residual flexibility persists which allows "swelling" to occur. In another study, conducted before the distinction between unfolding and swelling had been made clear, we characterized what we now recognize as swelling, by use of chemical probe techniques.[9] Employing a variety of contact-site cross-linking reagents,[7] we identified intranucleosomal "modal"[11] contacts between H2B and H4, which are disrupted as the nucleosomes swell at low ionic strength.[9] These disruptions occur regardless of the presence or absence of H1[8,9] consistent with the sedimentation assay.[8] We also described a specific intranucleosomal "skeletal"[11] contact between H2A and H2B, which is maintained throughout both unfolding and swelling.[9]

In this report we describe the use of iodination to characterize the unfolding response to low ionic strength. Focusing our attention on H4, we show that three tyrosines (#s 51, 88, and 98) are reactive to iodine in nucleosomes under conditions of moderate ionic strength (20-150 mM NaCl) but that the one remaining tyrosine in H4 (#72) is unreactive. In contrast, after nucleosomal unfolding at very low ionic strengths, the relative reactivities of these tyrosines become completely reversed. Moreover, indicative of this being a manifestation of the unfolding response, we show that H1 prevents this inversion of tyrosine reactivities at low ionic strength.

MATERIALS AND METHODS

Preparation of Chromatin Subunit Particles. Mononucleosomes and oligonucleosomes were prepared from calf thymus nuclei exactly as described previously.[12] The samples obtained in this way were dialyzed into 0.1 mM EDTA, 0.1 mM Tris HCl, pH 7.5, and stored frozen at -25°C at concentrations

greater than an A_{260} of 50. The presence of roughly equimolar amounts of the four core histones and the absence of detectable amounts of H1 were verified by analysis of the protein on both acid-urea gels (see Fig. 1) and Triton acid-urea gels (see Fig. 2). Electrophoresis of the nucleosomal DNA on a 4% polyacrylamide-$NaDodSO_4$ gel, according to the method of Todd and Garrard,[13] indicated that the DNA ranged from approximately 140-180 base pairs in length (not shown).

Chromatosomes were prepared according to the protocol of Simpson[2] exactly as described earlier.[8] In the present study, the material insoluble in 0.1 M KCl was resuspended in 1mM Tris, pH 8.0, dialyzed exhaustively against 0.1 mM sodium phosphate, 0.01 mM EDTA, pH 7.2,and stored frozen at -25°C at an A_{260} of 9.4. The size of the chromatosome DNA was determined by gel electrophoresis and found to be approximately 169$\pm$ 10 base pairs in length[8]. No attempt was made to remove the small amount of dinucleosome contamination revealed by this analysis. The presence of H1 along with the four core histones was verified by electrophoresis of the protein on a Triton gel (see Fig.8).

Iodination. Chromatin subunit particles were iodinated in a manner similar to that reported by Biroc and Reeder.[14] Subunit particles were diluted to an A_{260} of 2-4 with appropriate mixtures of 0.1 mM sodium phosphate, 0.01 mM EDTA, pH 7.6,and either 0.1 mM sodium phosphate, 0.01 mM EDTA, 20 mM NaCl, pH 7.6, or 0.1 mM sodium phosphate, 0.01 mM EDTA,200 mM NaCl, pH 7.6, to yield the desired concentration of NaCl. A 0.2 ml aliquot of this was transferred to a plastic microcentrifuge tube, 2 μl 10 mM chloramine-T, 1 mM sodium phosphate, pH 7.6, was spotted on one side of the tube, and 1 μl (20 μCi) ^{125}I (Amersham) in 0.01 M $NaHCO_3$, pH 8.0, was dotted on the other side of the tube. The tube was capped and vortexed gently to initiate the reaction which was in turn quenched after 20 minutes by the addition of 20 μl 50 mM sodium metabisulfite. After 5 min, 20 μl 2 M NaCl and 0.8 ml 95% ethanol were added, and the tubes were placed in a dry ice-ethanol bath for 30 min to precipitate the particles, which were collected by spinning for 15 min in an Eppendorf microcentrifuge. A 0.8 ml aliquot of 70% ethanol was added to the resultant pellet and, after 30 min in the dry ice bath, the tube was respun. The pellet was washed in this way a total of three times and finally allowed to air dry.

Polyacrylamide Gel Analysis. Nucleoprotein samples were analyzed directly on either acid-urea or Triton gels, using the protamine-release method of Shaw and Richards.[15] The gels were pre-electrophoresed and then scavenged at 125 volts for 1.5 hours with a solution of 8 M urea, 0.3 M cysteamine, 0.3 mg/

ml protamine, followed by a 0.3 hour scavenge at 125 volts with a solution of 8 M urea, 0.6 M cysteamine. The upper and lower reservoirs were filled with fresh 0.9 N acetic acid after each step. The pellets obtained from the ethanol precipitation were dissolved in loading buffer (8 M urea, 5% acetic acid, 2.5% thioglycolic acid, 5% β-mercaptoethanol, 0.1% protamine sulfate) to yield a final histone concentration of 1 µg/µl. Aliquots of 10-15 µl were then loaded onto the gels and electrophoresed as usual. For acid-urea gels, the method of Panyim and Chalkley[16] was used as previously described.[17] The Triton acid-urea gels were prepared according to the method of Urban, et al,[18] using 12% polyacrylamide gels which contained 6 mM Triton X-100 and 7.5 M urea.

Whereas we often prestain both acid-urea gels and $NaDodSO_4$ gels in 10% trichloracetic acid, 25% isopropanol for time periods governed by convenience only (but always greater than 0.5 h), we have found Triton gels to be offended by such cavalier treatment. Therefore, to prevent loss of histones from the Triton gels, we routinely prestain them for 0.5 h only.

Second-dimension $NaDodSO_4$ electrophoretic analyses of lanes from Triton gels which had been dried onto filter paper were exactly as described previously,[19] except that the rehydrated gel strips were soaked in 1% $NaDodSO_4$, 3 M urea, 0.11 M Tris, 10% β-mercaptoethanol, pH 7, for only 10 min instead of 1 h before imbedding them over the stacking gels. For second-dimension analyses on stacking acid-urea gels, we used the acid-urea-CTAB stacking gel procedure of Bonner et al,[20] with 20% acrylamide and 0.14% bis-acrylamide in the resolving gel. All two-dimensional gels were dried down and autoradiographed directly without staining for protein.

Peptide Mapping. Cyanogen bromide analyses were carried out on iodinated H4 as follows. Coomassie Blue bands corresponding to the non-acetylated form of H4 were excised from lanes of acid-urea gels and the protein eluted during an overnight incubation in 0.4 ml 40% thioglycolic acid, 0.9 N acetic acid. The supernatant was removed from each, and 0.2 ml acidified acetone (5N H_2SO_4: acetone, 1:500) was added to precipitate the protein. The pellets obtained after centrifugation were washed once with acidified acetone and allowed to air dry. The pellets were dissolved in 18 µl loading buffer (see above) which contained 1 µg/µl total unlabeled histone, and 15 µl of each was loaded onto a Triton gel. The gel was electrophoresed, stained, and destained. Using the stained carrier histone for reference, the bottom stained H4 band (containing "lower" labeled H4, see Results) and the third from bottom stained band (containing "upper" labeled H4) were cut from each lane and the protein eluted and precipitated as described above. The pellets were resuspended in 0.2 ml

40% thioglycolic acid in capped tubes and incubated at room temperature overnight to reduce any oxidized methionine residues. The samples were then precipitated with 5 volumes acidified acetone and washed two times with the same solution. Each pellet was resuspended in 0.1 ml 70% formic acid, which contained approximately 32 mg/ml freshly distilled CNBr. The tubes were capped immediately, and the digestion was allowed to proceed at room temperature in the dark for 16 hours. At this point, the samples were frozen, lyophilized over pellets of NaOH, resuspended in loading buffer, and electrophoresed on an acid-urea gel as described.[19] The gel was subsequently dried onto filter paper and autoradiographed.

Both zero- and mono-acetylated H4 were subjected to tryptic peptide analyses. The zero- and mono-acetylated forms of H4 were isolated, free of more highly acetylated forms, from an acid-urea gel and rerun on a Triton gel in order to obtain "lower" and "upper" H4 samples (see Results) without the latter being contaminated with highly acetylated species of normal mobility. The bands were eluted as follows, using a variation of the procedure of Bray and Brownlee.[21] After soaking the gel overnight in H_2O, bands were cut out and eluted at 65°C for a total of 3 h in three changes (0.2 ml of each) of 1 mM sodium phosphate, 0.2% $NaDodSO_4$, pH 7.6. The successive washes for each sample were combined, frozen, and lyophilized. The $NaDodSO_4$ was removed by extraction with a mixture of acetone: triethylamine: acetic acid: H_2O (85:5:5:5,v/v/v/v), as described by Henderson et al.[22] Each pellet was then resuspended in 0.1 ml of 0.1 M NH_4HCO_3, pH 8.0, and digestion was initiated by the addition of 10 μg TPCK-trypsin. Another 10 μg of trypsin was added during the course of the 24-hour incubation at 37°C. Following digestion, the samples were frozen, lyophilized, resuspended in 10 μl H_2O, and spotted onto 3 MM Whatman filter paper in 1 μl aliquots. The paper was wetted with a pH 3.5 buffer (10% acetic acid, 0.1% pyridine), electrophoresed at 2500 volts for 1.25 hours, dried, and autoradiographed.

RESULTS

Low Ionic Strength Induces an Altered Iodination Pattern in Nucleosomal H4. We have been engaged in elucidating the structural details of nucleosomal conformational changes which are induced by conditions of low ionic strength.[8,9] To identify tyrosine-containing histone domains which become perturbed as nucleosomes undergo these conformational changes, we have radioiodinated nucleosomes as a function of ionic strength.

As a first step in our analysis of the radioiodinated nucleosomes, we

electrophoresed the labeled nucleosomal histones on an acid-urea gel,[16] using the protamine-release method of Shaw and Richards.[15] The gel was stained and autoradiographed, and the results are shown in Figures 1A and 1B, respectively. H2A is found to be essentially nonreactive within isolated nucleosomes at the various ionic strengths tested, in agreement with results on chromatin obtained in other laboratories.[14,23] On the other hand, H2B and H3 are efficiently labeled at 150 mM NaCl but become less reactive as the ionic strength is reduced below 20-50 mM NaCl. H4 also is efficiently labeled at 150 mM NaCl, but, unlike the situation for H2B and H3, the amount of label incorporated into H4 is relatively invariant between 0 and 150 mM salt.

A surprising result was obtained when aliquots of the same histone samples were resolved on a Triton gel.[18] The autoradiograph shown in Figure 2B

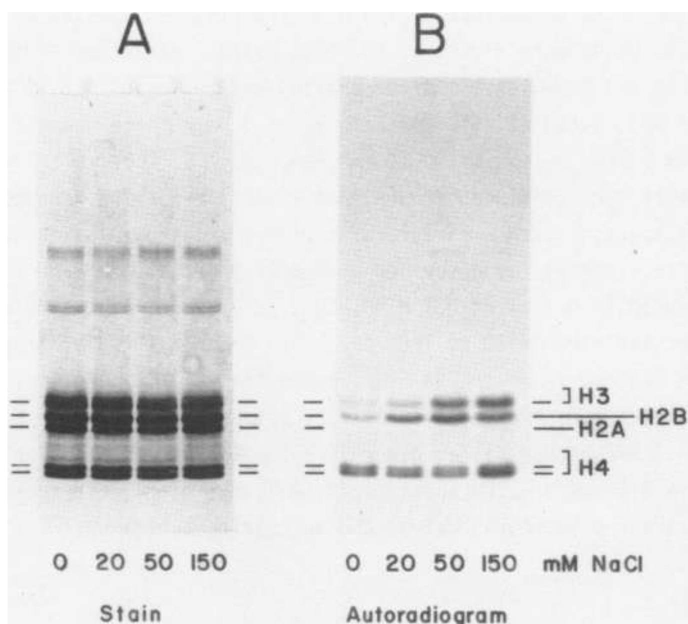

Figure 1. Acid-urea gel characterization of nucleosomal histones which have been iodinated *in situ*. Nucleosomes were iodinated in buffers containing 0.1 mM sodium phosphate, 0.01 mM EDTA, and either 0, 20, 50, or 150 mM NaCl as described in Materials and Methods. The samples were precipitated, resuspended in the protamine-release loading buffer of Shaw and Richards,[15] and aliquots were then loaded directly onto an acid-urea gel. The Coomassie Blue staining pattern for the acid-urea gel is shown in Figure 1A, and an autoradiograph of this gel is presented in Figure 1B.

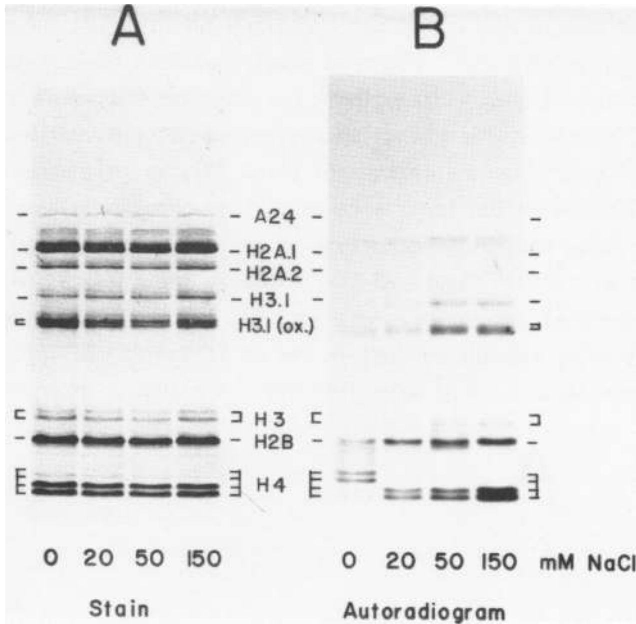

Figure 2. Triton gel characterization of nucleosomal histones which have been iodinated *in situ*. Aliquots of each of the samples analyzed on the acid-urea gel of Figure 1 were also electrophoresed on a Triton gel. The Triton gel was stained (Fig. 1A) and the particular histone variants identified by reference to characteristic patterns obtained in other labs.[18,24] Control experiments have demonstrated that the putative oxidized form of H3.1 results from the exposure of nucleosomes to Chloramine-T and is not dependent on modification by iodine (not shown). The autoradiograph shown in Figure 2B was obtained by exposing the Triton gel to film for the same amount of time (15 min.) as the acid-urea gel autoradiograph presented in Figure 1B.

reveals, in the H4 region of the gel, a dramatic qualitative difference between the sample iodinated at 0 mM NaCl and those iodinated at 20-150 mM NaCl. Although it appears that the 0 mM sample is distinct from the other three in having most of the label in highly acetylated species of H4, it is apparent from the acid-urea gel of Figure 1B that this explanation cannot be correct. The acid-urea gel shows that in all cases essentially no labeled H4 is present in its highly acetylated forms. The stained Triton gel pattern shown in Figure 2A demonstrates that the mobility of H4 as a whole is unchanged on the Triton gel. Therefore, the two labeled bands which migrate in the region of highly acetylated H4 on the Triton gel must correspond either to highly iodinatable but previously uncharacterized proteins (which turns out not to be the case) or to H4 molecules which migrate anomalously in the Triton gel system as a result of the iodination *per se* and,

moreover, as a result of the iodination having been carried out on nucleosomes at low ionic strength. Figure 3 shows that this ionic strength dependence is not obtained when isolated bulk histones are iodinated under similar conditions. Therefore, the change in iodination pattern, which is visualized in the H4 region of the Triton gel (Fig. 2B), is indicative of a *nucleosomal* event induced by low ionic strength. This phenomenon has been observed consistently using several preparations of nucleosomes, although it is not always as dramatic as in Figure 2B (for example, see Figs. 5 and 6).

Two-dimensional gel analysis was employed to confirm the identity of each of the labeled species present in the H4 region of the Triton gel. Oligonucleosomes devoid of H1 were iodinated in either 0 mM or 20-25 mM NaCl,

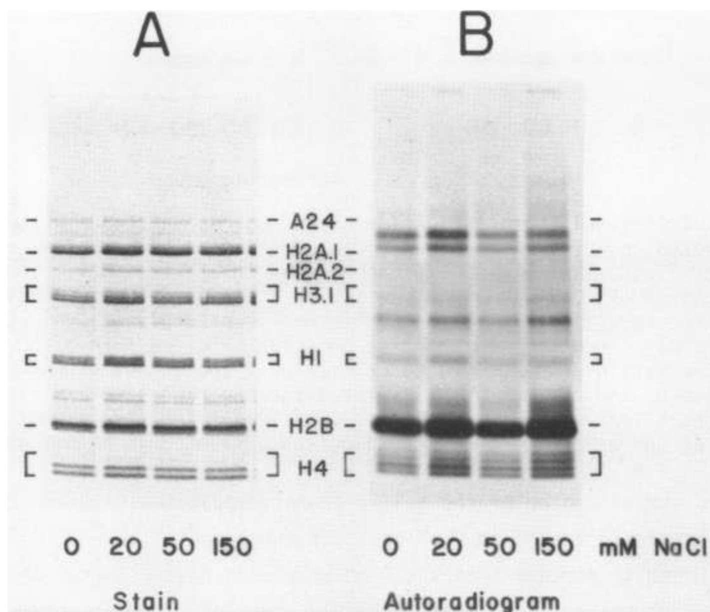

Figure 3. Free histones do not display a "flip-flop" in the H4 iodination pattern as a function of salt concentration. Bulk histones were isolated from nuclei by extraction with 0.4 N H_2SO_4 and precipitation with acetone as previously described.[25] The histones were resuspended in H_2O at a concentration of approximately 10 mg/ml, and the pH was adjusted to approximately 7.6 by the addition of 250 mM Na_3PO_4. This histone sample was diluted 50 fold into solutions containing 0.1 mM sodium phosphate, 0.01 mM EDTA, pH 7.6, and 0, 20, 50 or 150 mM NaCl. Aliquots (0.2 ml) were iodinated as described in Materials and Methods, and the samples were precipitated with one volume of 50% trichloracetic acid in the presence of 60 mg of carrier histone. The pellets were washed twice with acidified acetone,[19] resuspended in loading buffer, and 15 µl (15 µg) of each were electrophoresed on a Triton gel. The stained gel is shown in part A, and a 15 min autoradiogram is shown in part B.

the histones were electrophoresed in a Triton gel as in Figure 2, and then each lane was cut out, electrophoresed into a second dimension $NaDodSO_4$ gel, and autoradiographed (Fig. 4). The patterns obtained show that all of the different labeled species which have various mobilities within the H4 region of the Triton gel have the same mobility (i.e., that of H4) in the $NaDodSO_4$ gel.

To address the paradox concerning the acetylation status of the major labeled H4 doublet obtained when nucleosomes are iodinated under conditions of low ionic strength (Fig. 2B, left lane), another type of two-dimensional

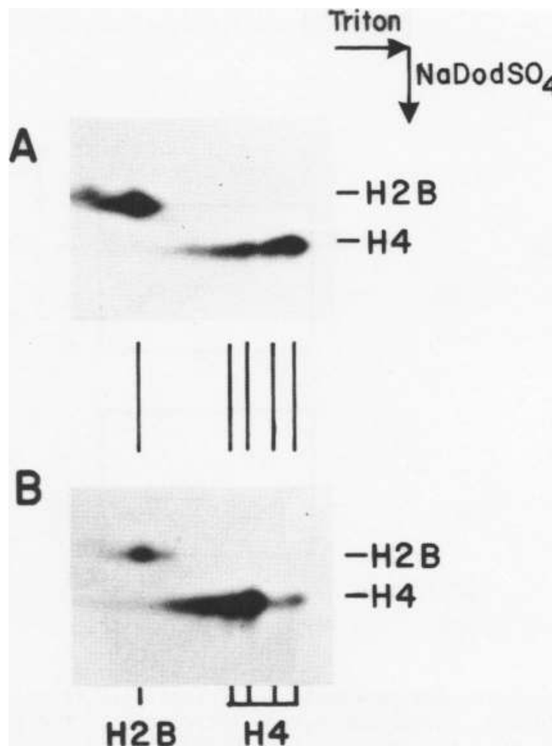

Figure 4. All of the labeled bands in the H4 region of the Triton gel are indeed species of H4. Nucleosomal histones were iodinated *in situ* in the presence of either 25 mM (panel A) or 0 mM (panel B) NaCl and electrophoresed on a Triton gel. When the gel was stained, dried on filter paper, and autoradiographed, results were obtained which were comparable to the data presented in Figure 2 (not shown). The two lanes were then excised and the protein electrophoresed into second dimension $NaDodSO_4$ gels as described in Materials and Methods. The gels were autoradiographed, and the H4 regions of each are shown.

gel analysis was carried out, this time using acid-urea-CTAB in the second dimension as described by Bonner et al.[20] The H4 regions of the autoradiographs are shown in Figure 5, again for samples iodinated in 0 or 20 mM NaCl. Since the mobility of H4 is reduced in proportion to its degree of acetylation in both the Triton and acid-urea-CTAB dimensions, the non-iodinated acetylated species of H4 all fall on a diagonal in this type of gel (not shown, see Ref. 20). However, the autoradiographs reveal that the *iodinated* species of H4 lie on *two adjacent* diagonals, and that the labeled doublet which migrates in the position of highly acetylated H4 in the Triton dimension is actually predominantly zero- and monoacetylated H4 as revealed by its migration in the acid-urea dimension. This is best illustrated by the anomalously reduced mobility in only the Triton dimension of almost all of the labeled H4

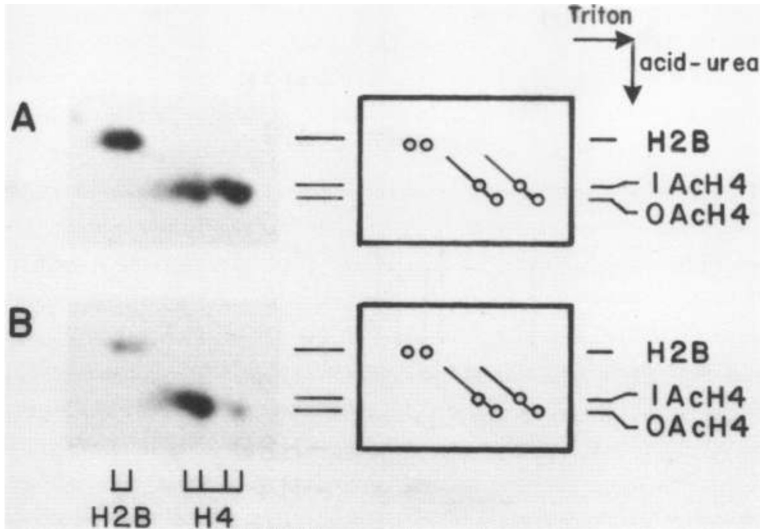

Figure 5. The upper labeled H4 doublet on Triton gels is primarily zero- and mono-acetylated H4. Nucleosomes were iodinated and the histones resolved in a first-dimension Triton gel to yield stained and labeled patterns similar to Figure 2 (not shown). Two lanes were cut out from this Triton gel and subjected to electrophoreses in a second-dimension acid-urea gel. Autoradiographs of the H4 regions are shown for the two-dimensional gels obtained using nucleosomes iodinated in 20 mM NaCl (part A) and 0 mM NaCl (part B). About half the H2B migrates in the first-dimension Triton gel in the position of oxidized H2B, both in the labeled pattern (this figure) and in the stained pattern (not shown). Usually the reduced form of the H2B is regenerated when sodium metabisulfite is added to quench the iodination reaction. In this experiment, however, the reaction was terminated by dialysis in the presence of excess tyrosine.

in the sample iodinated in 0 mM NaCl (Fig. 5B). We will now show that this altered mobility on Triton gels is a consequence of the iodination of one particular tyrosine residue within H4 which becomes reactive in nucleosomes under conditions of low ionic strength.

Iodination at Tyrosine 72 Causes H4 to Migrate with Reduced Mobility on Triton Gels. Since the creation of an iodotyrosine residue in place of tyrosine is, in effect, an amino acid replacement which does not alter the overall charge of the molecule, the iodinated H4 species should comigrate with the stained bands on acid-urea gels, as is observed. Triton gels, on the other hand, can resolve histones which differ by as little as a single neutral amino acid, provided the substitution falls within a "Triton-sensitive" region of primary sequence.[18] Thus, the observation that in some samples a subset of labeled H4 migrates on Triton gels with a mobility distinct from that of unlabeled H4 suggests that at least one tyrosine residue lies within such a "Triton-sensitive" region of primary sequence. Similarly, the fact that in other samples a pair of labeled H4 bands co-migrates with the major stained doublet indicates that at least one tyrosine must lie outside the "Triton-sensitive" regions. According to this reasoning, the set of tyrosine residues which is found to be iodinated in the H4 of reduced mobility on Triton gels (which we will refer to as "upper" H4) should contain at least one residue which is not found labeled in the H4 species of unaltered mobility (which we will call "lower" H4).

To identify which tyrosine residues are iodinated in each case, upper H4 and lower H4 were separately isolated from Triton gels and subjected to two types of peptide analysis. As a preliminary characterization, we digested each sample with cyanogen bromide and resolved the resultant N-terminal and C-terminal fragments from the undigested H4 on an acid-urea gel. An autoradiogram of this gel is shown in Figure 6, where the digested lower H4 was loaded in lanes 1 and 3 and the digested upper H4 appears in lanes 2 and 4. (The H4 in lanes 1 and 2 was obtained from nucleosomes iodinated in 0 mM NaCl, whereas the samples in lanes 3 and 4 derived from nucleosomes iodinated in 10 mM NaCl.) The digested upper H4 sample yields label in only the N-terminal fragment (lanes 2 and 4), showing that tyrosine 51 or (and) 72 is (are) in a "Triton-sensitive" domain. Moreover, since the lower H4 sample can also have label in the N-terminal fragment (lane 1), we know that only one of the two tyrosines in this fragment can be within the "Triton-sensitive" region of primary sequence responsible for the altered mobility. There is no evidence for either of the C-terminal tyrosine residues being in a "Triton-sensitive"

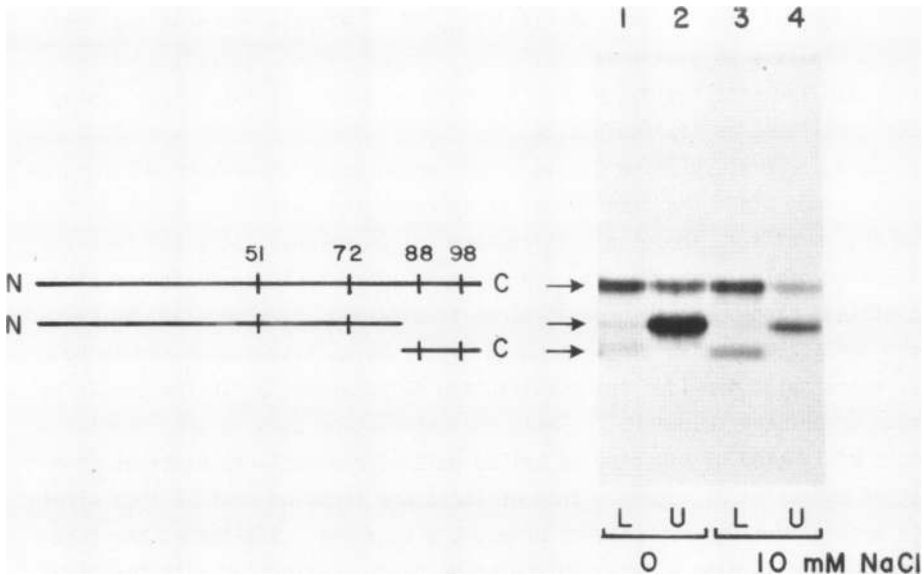

Figure 6. Iodination at a specific tyrosine residue reduces H4 mobility on Triton gels. Iodinated mono-acetylated H4, isolated from an acid-urea gel, was resolved on a Triton gel into an upper band (of reduced mobility) and a lower band (of normal mobility). Each band was isolated separately, subjected to CNBr digestion, analyzed on an acid-urea gel, and autoradiographed. Lanes 1 and 2 contain the lower and upper H4 species, respectively, obtained from nucleosomes iodinated in 0 mM NaCl. Lanes 3 and 4 contain the lower and upper H4 species, respectively, obtained from nucleosomes iodinated in 10 mM NaCl. The positions of the tyrosine residues within H4, and their segregation to the two fragments generated when H4 is cut by CNBr at the single methionine residue at position 84,[26] are depicted schematically to the left of the autoradiogram. As indicated in this figure, the top band is residual undigested H4, the middle band is the large N-terminal fragment, and the bottom band is the small C-terminal fragment.

region.

In order to identify which of the two N-terminal tyrosine residues, 51 or 72, causes the reduced H4 mobility on Triton gels as a result of iodination, we followed the procedure for tryptic peptide analysis described by Biroc and Reeder.[14] Trypsin is particularly useful in this regard because it cleaves H4 such that each of the four tyrosines is contained within a distinct peptide, and, moreover, these tyrosine-containing peptides can be resolved in an ordered fashion using one-dimensional paper electrophoresis.

The autoradiograph shown in Figure 7B presents the data obtained when lower H4 and upper H4 were separately excised from the same lane of a Triton gel and analyzed as shown schematically in Figure 7A. The middle horizontal

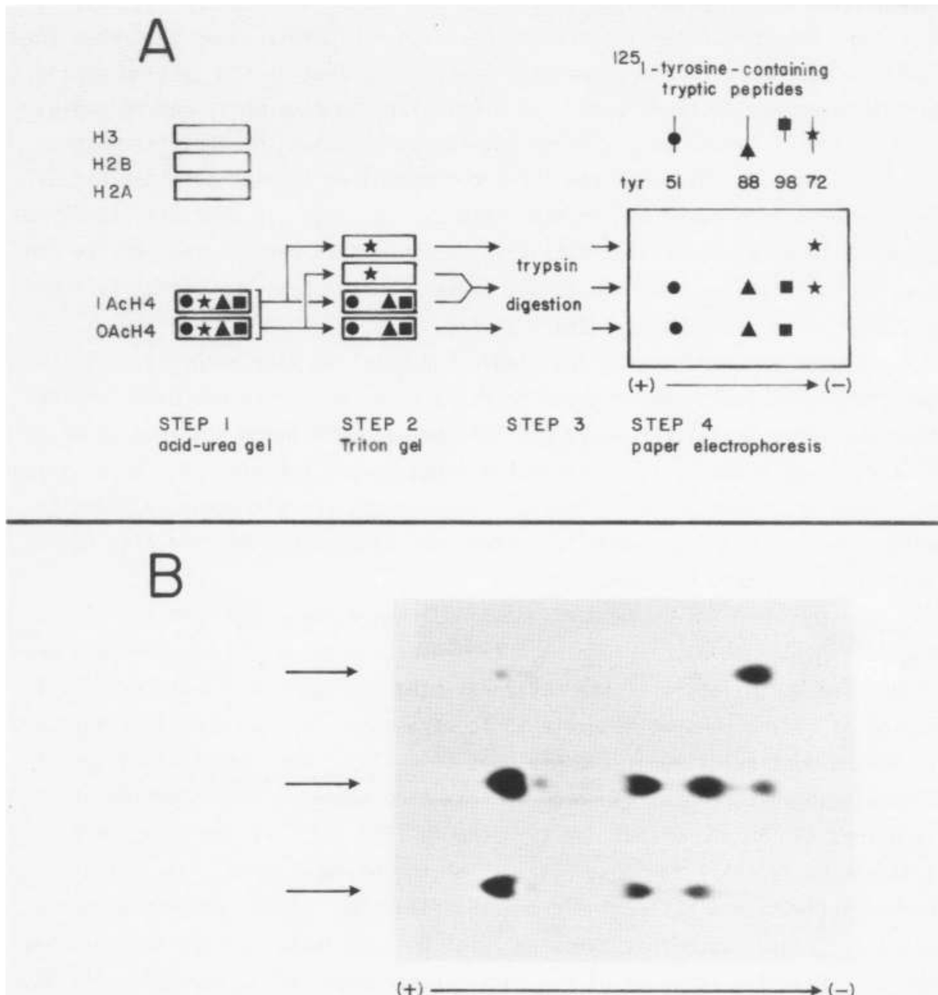

Figure 7. Iodination at tyrosine 72 is responsible for the reduced H4 mobility on Triton gels. Tryptic peptide analysis of the two classes of labeled H4 (obtained from nucleosomes iodinated in 8 mM NaCl) was carried out as outlined schematically in part A. The non- and mono-acetylated H4 doublet was excised from an acid-urea gel (step 1) and rerun on a Triton gel (step 2). This resolves the single acid-urea doublet into the two Triton doublets--the lower doublet of normal mobility and the upper doublet of reduced mobility. Bands were excised and digested with trypsin (step 3), electrophoresed on paper at pH 3.5 (step 4) and then autoradiographed. Note that the middle horizontal lane (step 4) is a mixture of digested upper and lower H4 and serves to mark the positions of the four tyrosine-containing tryptic peptides. These have been identified by Biroc and Reeder[14] as diagrammed in part A. The actual autoradiogram from step 4 is shown in part B.

lane contains both classes of labeled H4 and serves to mark the positions of the four tyrosine-containing tryptic peptides. Figure 7 shows that when the lower H4 and upper H4 components are analyzed separately the labeled peptides partition as two distinct sets. In particular, H4 apparently can be iodinated at any of tyrosines 51, 88 or 98 without altering the mobility of the resultant labeled H4, since the three corresponding labeled peptides can all be recovered from lower H4 (bottom track in Fig. 7B). In contrast, tyrosine 72 must lie within a "Triton-sensitive" domain of H4 because the peptide containing this labeled tyrosine is not present in lower H4 but rather is found exclusively in upper H4 (top track in Fig. 7B).

We note parenthetically that iodinated H2A.1 is also resolved on Triton gels into two bands, one of which co-migrates with the stained band, whereas the other migrates with reduced mobility between H2A.1 and A24 (not shown). We also suspect that various iodinated forms of H2B can similarly be resolved on Triton gels (see below). However, we have not yet attempted to identify which tyrosine residues, when iodinated, are responsible for the altered mobilities of these histones.

The Low Ionic Strength Conformational Change "Exposes" Tyrosine 72 and "Buries" Tyrosines 51, 88, and 98. The results obtained in the previous section allow us to interpret the data presented in Figure 2. Thus, radioiodination of nucleosomes at moderate ionic strengths (20-150 mM NaCl) reveals H4 to be the most reactive of the histones (Fig. 1B). The reactivity of H4 at moderate ionic strengths is predominantly a consequence of iodination at tyrosines 51, 88 and 98 but *not* tyrosine 72 (Fig. 7). At very low ionic strength H4 is still the most reactive of the histones (Fig. 1B), but this high reactivity now reflects a dramatic "flip-flop" of H4 tyrosine accessibilities (Fig.7) such that tyrosine 72 of H4 (previously unreactive) becomes the most reactive tyrosine of the entire nucleosome, while tyrosines 51, 88, and 98 become relatively unreactive. Since iodination of tyrosine 72 but not tyrosines 51, 88 or 98, reduces the mobility of H4 in Triton gels (previous section), the "flip-flop" in tyrosine accessibilities is manifested as a "flip-flop" in the Triton gel autoradiographic pattern (Fig. 2B).

The Presence of H1 Blocks the Low Ionic Strength "Flip-Flop". We have shown previously that H1 blocks the major abrupt low ionic strength conformational change of nucleosomes[8] but that it does not block an independent swelling response which nucleosomes also exhibit at low ionic strength and which can be assayed by both physical[8] and chemical[9] techniques. To determine whether H1 would block the low ionic strength response of nucleosomes which

is detected by radioiodination, we probed H1-containing nucleosomes at 0 and 20 mM NaCl using the iodination-Triton gel protocol described above for H1-depleted nucleosomes. Chromatosomes[2,8] were chosen for this analysis because they are the simplest of such particles. Following iodination, chromatosomal histones were resolved by electrophoresis on a Triton gel and autoradiographed as shown in Figure 8. In contrast to the data presented in Figure 2 for H1-depleted nucleosomes, the majority of the H4 label in Figure 8 comigrates with the H4 stained bands, even for the chromatosome sample which was iodinated in 0 mM NaCl. Based on our characterization of the Triton gel presented in the previous section, these data show that H1 prevents tyrosine 72 from becoming reactive in chromatosomes under conditions of low ionic strength. Moreover, since H4 continues to be labeled at very low ionic strength, we conclude that H1 also serves to maintain the other H4 tyrosines in their re-

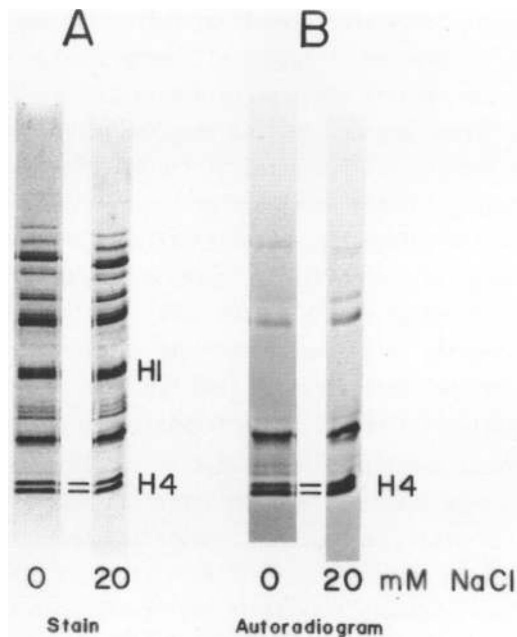

Figure 8. Nucleosomes which contain H1 do not display the low ionic strength "flip-flop". Chromatosomes were iodinated in solutions of 0.1 mM sodium phosphate, 0.01 mM EDTA, and 0 or 20 mM NaCl, pH 7.6, and analyzed in exactly the same manner as described previously for H1-depleted nucleosomes (see Materials and Methods). The iodinated chromatosome histones were resolved on a Triton gel and stained (part A) and then autoradiographed (part B). Note the presence of H1 in the stained gel.

active states. This stabilizing effect of H1 occurs despite the slightly greater average length of the chromatosomal compared to the nucleosomal DNA (see Materials and Methods) a difference which, by itself, would exert a destabilizing influence.[8] Thus, the presence of H1 stabilizes nucleosomes such that an important structural feature of the C-terminal region of H4 is not disrupted under conditions of low ionic strength.

DISCUSSION

Conformational changes of the nucleosome histone core at low ionic strength. The data we have presented show that H4 can exist in two different and well defined conformational states in the nucleosome, depending on the ionic strength. H4 has four tyrosines. At physiological ionic strength, the conformation is characterized by availability of tyrosines 51, 88 and 98, but not 72 for iodination. The low ionic strength conformation is characterized by availability of tyrosine 72 for iodination but not tyrosines 51, 88 or 98. In our iodination studies only trace levels of iodination have been employed so as to minimize the occurrence of potentially harmful side reactions. However this means that our results might pertain only to a specific subpopulation of nucleosomes. These may be imagined to arise during preparation or they could represent intrinsically special particles. Nevertheless even without specific knowledge of the exact proportion of the population being assayed the nature of the information obtained allows for a well defined interpretation of the results. Accordingly, based on arguments to follow, we believe that tyrosine 72 marks a stable structural domain of the histone core which participates directly in the major discrete unfolding process which nucleosomes undergo at low ionic strength (see Ref. 8).

The inaccessibility of tyrosine 72 to iodination in nucleosomes at physiological ionic strength and the altered mobility of tyrosine 72-iodinated H4 on Triton gels both argue that this residue is folded into a tight, probably hydrophobic, domain in native nucleosomes. Regarding the inaccessibility of tyrosine 72 to iodination at physiological ionic strength, the most straightforward interpretation is simply that it is physically protected within the nucleosome. Although this is not the only possibility, the altered mobility on Triton gels of H4 iodinated at tyrosine 72 strongly supports such an interpretation by the following logic. First we note that it is not iodination *per se* which is important to the mobility change on Triton gels. Rather it is the domain of H4 into which the iodine is inserted that is critical. Since reduced mobility presumably reflects alterations in hydrophobic domains

of particularly stable residual secondary structure in this denaturing gel system,[35,36] tyrosine 72 apparently resides in such a domain. Further, we note that the only tyrosine residue to be influential with regard to H4 mobility on Triton gels is the very same tyrosine which, alone, is exposed upon decreasing the ionic strength. This presumably reflects the exposure of an internal hydrophobic nucleosomal domain as a result of the stress of low ionic strength. Since this conformational change is blocked by H1 (see below) it is probably part of the abrupt "unfolding" response to low ionic strength as previously defined.[8]

Our iodination results have shown not only that tyrosine 72 is rendered accessible when nucleosomes are subjected to conditions of low ionic strength, but also that tyrosines 51, 88 and 98 are rendered *in*accessible by the same treatment. Therefore, the unfolding which exposes tyrosine 72 is likely a complex process which results in widespread conformation adjustments in the nucleosome. In this way it resembles the independent gradual "swelling" process which nucleosomes also undergo at low ionic strength,[8] and which results in the concurrent rupture of old and the establishment of new histone-histone contacts as the ionic strength is reduced.[9] (We should point out that our prior cross-linking studies[9] preceded the hydrodynamic studies[8] which resolved the gradual and abrupt responses of nucleosomes to low ionic strength. Therefore, in Ref.9, we speak in terms of "unfolding" exclusively).

H1 stabilizes the histone core. When nucleosomes containing H1 are exposed to very low ionic strength, there is no change in the iodination pattern of H4 (Fig.8). This is a significant observation because in the nucleosome, H1 probably does not contact H4 directly.[27-30] Based on the model of Klug et al[27] along with the probable placement of H1 on one side of the nucleosome[4,28] and the demonstrated orientation of H4 in the nucleosome (with its C-terminal domain in contact with H2B[19]), we deduce that the tyrosines of H4 are very nearly on the opposite side of the nucleosome from the site of H1 binding. Therefore, consistent with our previous hydrodynamic results[8], we conclude that H1 serves to stabilize the entire nucleosome particle by virtue of binding a critical region located on one side.

We note parenthetically that the presence of H1 on nucleosomes also has an effect on the iodination pattern of H2B. Thus as can be seen in Figure 8B, labeled chromatosomal H2B migrates above the stained H2B on Triton gels, whereas labeled H2B from H1-depleted nucleosomes (Fig. 2B) essentially co-migrates with the stained H2B band. Therefore, in addition to stabilizing nucleosome structure, H1 also imposes on the particle elements of structure

unique to the H1-bound state.

In a previous iodination study Griffiths and Huang[31] obtained patterns which appear to be a composite of our low ionic strength iodinations, plus and minus H1. Possibly their extensive chromatin preparation procedure led to redistribution of H1 away from some segments of chromatin. Alternatively, perhaps the erythrocyte H5-containing chromatin which they used is different from calf thymus chromatin. In a different kind of study Lewis[32] has previously presented data also suggesting that the C-terminal region of H4 is perturbed within nucleosomes at low ionic strength. However, in his study the site being assayed (methionine 84 of H4 which had been chemically derivatized) was actually perturbed at the outset of the experiment, so the data are difficult to assess. Other studies which have been carried out on derivatized reconstituted particles should also be interpreted with caution, since such chemical modifications of the histones have been shown to lead to destabilization of the resulting nucleosomal particles as pointed out by Lewis.[33]

It is noteworthy that the tyrosines of H4, which we find to be obligatorily involved in the unfolding process, are in the same region of H4 sequence as that which we have shown previously to be in contact with H2B.[19] Perhaps this reflects a natural tendency of the nucleosome to initiate the process of unfolding by lifting the H2A-H2B histone pairs on each side upwards and away from the H3-H4 tetramer, which may be considered to form a foundation (see Fig.7 of Klug et al[27]). This interpretation is supported by our previous results which showed that neither swelling nor unfolding[9] ruptures the H2A-H2B contact.[34] Current models for H1 binding[4,28] would predict that movement of H2A and H2B in this way, as a dimer unit attached to the DNA, would not be possible in the case of H1-bound nucleosomes in which the terminal segments of the DNA are constrained.[4] Accordingly, we have now shown by both physical (Ref. 8) and chemical (this report) techniques that H1 does indeed completely block unfolding of nucleosomes in solution at low ionic strength.

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FOOTNOTE

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