# Role of Individual Histone Tyrosines in the Formation of the Nucleosome Complex†

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ABSTRACT: We have determined the accessibility of histone tyrosine residues to react with *p*-nitrobenzenesulfonyl fluoride (NBSF) in intact nuclei, salt-dissociated nucleosomes, isolated histone complexes, and individual core histones. Of the 15 core histone tyrosine residues, 13 are inaccessible in native nucleosomes; only Tyr121 near the C-terminus of H2B is fully accessible, and Tyr54 of H3 is partially accessible under near-physiological conditions. When H1 and the basic N-terminal tails of the core histones are dissociated from the DNA by treating nuclei with 0.4 and 0.8 M NaCl, the two tyrosines which are adjacent to the basic regions of H2B and H3 become accessible as well. This indicates that these tyrosine residues may be involved in histone–DNA interactions, either directly or indirectly. When the H2A–H2B dimers are dissociated from the chromatin by raising the NaCl concentration to 1.2 M, three to four tyrosines located in the structured regions of H2B and H4 are exposed, suggesting that these tyrosine residues may be located at the dimer–tetramer interface. Dissociating all the histones from the DNA at an even higher ionic strength as a mixture of dimers, tetramers, and octamers does not change the pattern of Tyr exposure, but reduces the reactivity of the tyrosines at the dimer–tetramer interface as would be expected from the reassociation of H2A–H2B dimers and H3–H4 tetramers. The fact that our results with native nucleosomes in intact nuclei are in every aspect consistent with the crystallographic structure proposed for the DNA-free histone octamer of Arents et al. [(1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 10148–10152] indicates that the structure of the protein core complex is not affected by removal of the DNA at high ionic strength. The exposure to NBSF modification of histone tyrosines at the histone–DNA and the dimer–tetramer interface may be a useful indicator for nucleosome unfolding which has been postulated to occur during chromatin transcription and replication.

The genomes of all eukaryotes are organized into nucleosomes by the stable interaction of the DNA with the highly conserved histone proteins [for a comprehensive review of chromatin, see van Holde (1988)]. The four core histone species, which have a similar architecture but no amino acid sequence homology, interact with each other under physiological conditions to form H2A–H2B dimers and H3–H4 tetramers. The H3–H4 tetramer apparently binds first to DNA, followed by the binding of an H2A–H2B dimer on either side to form an octameric histone complex around which 165 base pairs of DNA are wrapped in approximately two superhelical turns (Hansen & van Holde, 1991). The fifth histone species (H1, H5) is thought to bind to the outside of the DNA superhelix across the two turns and into the DNA regions linking adjacent nucleosomes (Thoma et al., 1979). The nucleosome complex most likely has multiple functions. One of the primary functions is to package large genomes into chromosomes of a size manageable by the mitotic and meiotic genome segregation systems. The compact state is also useful for long-term protection from damage of DNA in long-lived nondividing cells, especially germ cells. Because the histones are designed to bind to any DNA sequence, they are universal repressors which compete with regulatory proteins for DNA binding sites. To gain access to the genetic information in DNA, the nucleosomes are thought to be unfolded or removed during transcription and replication (Thoma, 1991; van Holde et al., 1992). This may occur in several stages, although the detailed mechanisms are not known. Transcriptionally competent genes are located in chromatin domains in which the DNA is more accessible to nucleases (Weintraub & Groudine, 1976; Lawson et al., 1982). This altered chromatin conformation of a chromosomal domain is maintained by as yet unknown mechanisms. The enzyme complexes that transcribe or replicate DNA are associated with accessory factors necessary for unfolding DNA packaged into nucleosomes. Those factors have not been identified. The fate of the nucleosomes during transcription and replication is still controversial because conflicting data have been obtained in different model systems [reviewed by Thoma (1991) and van Holde et al. (1992)]. Cross-linking experiments showed that the histones are not removed from the transcribed regions of DNA but that their mode of interaction with the DNA is changed (Nacheva et al., 1989). Unfolding of the nucleosomes in transcribed regions was reported to result in a split nucleosome structure (Lee & Garrard, 1991) and in the unfolding of the H3–H4 tetramer with exposure of Cys110 of histone H3 (Chen et al., 1990) which is normally buried at the center of the nucleosome structure. Several models have been proposed for a stable altered nucleosome structure of active chromatin domains and for disassembly and reassembly of nucleosomes during transcription and replication (Nacheva et al., 1989; Jackson, 1990; Morse, 1992; van Holde et al., 1992). To test these models and to study the changes in the nucleosome structure during transcription and replication in more detail, it would be useful to have a probe for the status of the different binding sites involved in the formation of the nucleosome complex. The interaction of proteins with other macromolecules involves a significant portion of their surface. By comparing the pattern of exposed amino acids in the free and the complexed form of a protein, one can map

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the components of the binding site. We call this protein footprinting. There are a number of chemical reactions which are useful for probing the surface exposure of specific amino acids. Tyrosines, because of their size and their chemical versatility, are often found in critical morphogenic sites in proteins and their binding sites. All four core histones have one to three conserved tyrosines at the junction between the unstructured, basic N-terminal tails and the highly structured central regions. Some of these tyrosines could be involved in histone–DNA interactions and others in protein–protein interactions within the nucleosome complex. In the experiment reported here, individual tyrosine residues were mapped to specific molecular interaction sites of the core histones on the basis of their reactivity with *p*-nitrobenzenesulfonyl fluoride (Liao et al., 1982) in chromatin exposed to different salt concentrations which result in the stepwise dissociation of the nucleosome complex (van Holde, 1988).

## MATERIALS AND METHODS

*Reagents.* General laboratory reagents were at least analytical grade. *p*-Nitrobenzenesulfonyl fluoride (NBSF), thiodiglycol, aprotinin, leupeptin, Triton X-100, and Na-Hepes were from Sigma Chemical Co. Micrococcal nuclease and endoprotease Glu-C were from United States Biochemical Co., trifluoroacetic acid was from Pierce Chemical Co., and acetonitrile was from EM Industries.

*Isolation of Nuclei.* Calf thymus was obtained fresh from a local slaughterhouse, trimmed, quickly frozen on dry ice in small pieces, and stored in sealed containers at −70 °C until used. The frozen tissue pieces were homogenized in 5–10 volumes of ice-cold buffer I (20 mM Na-Hepes, pH 7.5, 10 mM sodium butyrate, 100 mM NaCl, 3 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.3% thiodiglycol, 300 KIU/mL aprotinin, and 1 μg/mL leupeptin) using either a small Waring blendor with foam adapter (25–50 mL) or an Ultraturrax homogenizer with foamless microprobe (1.2–5 mL). The homogenate was then filtered through a 37-μm nylon screen (Tetko, Inc., Briarcliff, NY) into polycarbonate centrifuge tubes and underlayered with an equal volume of buffer I diluted with 1 volume of 0.5% Triton X-100 and 10% ultrapure sucrose. The nuclei were pelleted by centrifugation at 2000 rpm for 3 min in an HB-4 rotor using a Sorvall RC–5B refrigerated centrifuge. The supernatant was removed by aspiration and the nuclear pellet homogenized in 5 mL/g of tissue of buffer I diluted with an equal volume of water. Because the clean, detergent-treated nuclei adhere to each other, moderate force is sufficient to break most nuclei. The nuclear fragments do not clump and provide a homogeneous substrate for subsequent reactions. This is especially important in comparative studies with nuclei at different salt concentrations. Aliquots of the nuclear homogenate were diluted 200-fold with 1 M NaOH, and the amount of DNA was estimated from the absorbance at 260 nm. The volume of the nuclear homogenate was adjusted to approximately 1 mg/mL DNA. In most experiments where nuclei were treated in high salt, the viscosity was reduced by first digesting the nuclear homogenate with 80 units/mL micrococcal nuclease for 10 min on ice and then stopping the reaction by adding Mg-EGTA, pH 7.8, to 2 mM.

*NBSF Treatment and Isolation of the Histone Proteins.* NBSF was added to nuclear homogenates to a concentration of 2 mM from a fresh 100 mM stock solution in 2-propanol, and incubated in a 24 °C water bath for 10 min. The reaction was stopped by adding HCl to 0.3 N and either NaCl to 1 M or $CaCl_2$ to 0.5 M. After 10 min on ice, the acid-insoluble material was removed by centrifugation at 10000*g* for 5 min. The histones were recovered from the supernatant by precipitation with 20% TCA. The precipitate was collected by centrifugation as above and washed consecutively with (a) 20% TCA, (b) 0.4% HCl and 1% thiodiglycol in acetone, and (c) 1% thiodiglycol in acetone. The acetone was removed from the final pellet with dry air, and the histones were dissolved in distilled water at ca. 2 mg/mL protein.

*HPLC Separation of the Histones.* The histone proteins were resolved by reversed-phase chromatography on 5-μm Bakerbond Widebore C8 resin (J. T. Baker Co.) in either a 4.6 × 250 mm or a 10 × 250 mm column. To optimize the resolution of all the variants and their NBSF-modified forms, the elution conditions were adjusted by changing ion pairing while maintaining a high enough ionic strength to minimize nonspecific adsorption of the very basic and arginine-rich histone proteins. This was achieved by adjusting the concentrations of trifluoroacetic acid, acetic acid, and $CaCl_2$. $Ca^{2+}$ is a strong competitor for anionic binding sites on metal and glass surfaces while at the same time being very soluble in alcohols and acetonitrile. The optimal conditions for the specific resin batch used in these experiments were 20 mM $CaCl_2$, 0.1% acetic acid, 25% acetonitrile, and 3.5% methanol as solvent A and 20 mM $CaCl_2$, 0.1% acetic acid, 70% acetonitrile, and 10% methanol as solvent B with the following gradient program: 0–10 min, linear gradient from 0 to 33% B; 10–30 min, a slightly concave gradient (Waters gradient program 7) from 33 to 80% B, followed by a brief wash in 100% B before reequilibration. The column was maintained at 37 °C, and the elution rate was 1.4 mL/min for the 4.6-mm column and 3.5 mL/min for the 10-mm column. The eluate was monitored at 214 nm for quantitation of proteins and at 254 nm for identification of NBSF modifications. Peaks of interest were collected manually. After the organic solvents had been removed in a vacuum centrifuge, the histone fractions were precipitated with 20% TCA and recovered as described above.

*HPLC Peptide Mapping of Histones.* Individual or total histones were dissolved in 50 mM sodium formate, pH 4.0, at 2 mg/mL and digested with 0.1 mg/mL endoproteinase Glu-C for 16–24 h at 37 °C. The peptides were resolved by reversed-phase chromatography on the same 5-μm Bakerbond Widepore C8 resin described above. The solvents in this case were as follows: A, 0.2% trifluoroacetic acid; (B) 0.2% trifluoroacetic acid in 80% acetonitrile. The elution was performed with a linear gradient from 0 to 30% B in 9 min, a linear gradient from 30 to 43% B in 11 min, and a convex gradient (Waters 5) from 43 to 100% B in 4 min. The eluate was monitored at 214 nm for monitoring all peptides, at 254 nm for the detection of NBSF modifications, and at 280 nm for identification of tyrosine-containing peptides.

*Amino Acid Analysis.* Peptides were hydrolyzed for 18 h at 110 °C in HCl vapors in the presence of phenol, derivatized with phenyl isothiocyanate, and the PTC-amino acids were resolved by reversed-phase chromatography according to the PicoTag protocol of the Waters Division of Millipore Corp., with minor modifications, by the Protein Analysis Facility of the Fox Chase Cancer Center.

*Isolation of Native Histone Complexes.* Calf thymus nuclei digested with micrococcal nuclease as described above were lysed in 1.6 M NaCl, 20 mM Na-Hepes, pH 7.5, 0.3% thiodiglycol, 300 KIU/mL aprotinin, and 2 μg/mL leupeptin. Insoluble material was removed by centrifugation at 10000*g* for 5 min. The supernatant was mixed with an equal volume of 10 mg/mL protamine hydrochloride, pH 7.5, to precipitate the DNA. After 10 min on ice, the precipitate was removed by centrifugation at 18 000 rpm (33000*g*) for 5 min in a Sorvall SM24 rotor. The supernatant was then dialyzed against and

fractionated on a 0.9 × 100 cm Superose 12 (Pharmacia LKB Biotechnology) column in 10 mM Tris–phosphate, pH 7.4, and 1 mM DTT containing either 2.4 M NaCl for the isolation of histone octamers or 0.14 M NaCl for the isolation of H3–H4 tetramers and H2A–H2B dimers. The column was calibrated with alcohol dehydrogenase, serum albumin, carbonic anhydrase, and cytochrome *c*.

## RESULTS

*Protein Footprinting Strategy.* Protein footprinting compares the solvent exposure of the amino acid side chains of a protein in the free form and in complexes by reacting exposed hydrophilic groups with small chemical reagents. A fundamental requirement for a footprinting method is that a significant fraction of the accessible sites can be reacted without causing alterations in the structure of the protein or the complexes it forms. The usefulness of a footprinting method can be judged by the difference in reactivity between protected internal and exposed surface residues. In order to avoid even transient unfolding of the protein, footprinting reactions have to proceed rapidly under near-physiological conditions of pH and ionic strength. As demonstrated below, the reaction of tyrosine residues with NBSF (Liao et al., 1982) fulfills these conditions. One important advantage of footprinting methods is that the nature of native complexes can be assessed by "mapping" the surface of protein complexes in situ, i.e., in very complex systems such as whole tissue homogenates or intact nuclei, before purifying the protein. One can then compare the "native" state of the protein in the cell with that of the purified protein and reconstituted complexes. In a typical experiment, a crude homogenate as well as purified proteins is treated under the same conditions with the reagent. The reactions are stopped by inactivating the reagent. Individual proteins are then purified and digested with appropriate endoproteases, the peptides are isolated by HPLC-fingerprinting, and their identity is established by amino acid analysis. The degree of modification can be established by spectroscopy, by determining the specific radioactivity, or by amino acid sequencing. In the case of tyrosine modification with NBSF, which results in the introduction of a chromophore as well as a change in retention on reversed-phase chromatography resins, the degree of modification can be established directly by spectroscopic quantitation of the resolved modified and unmodified forms of the proteins and/or the peptides during reversed-phase HPLC.

*Preliminary Kinetic Experiments.* We first established that only 2 of the 15 different core histone tyrosines could be reacted in intact nuclei. No other tyrosine modification was detectable even in prolonged incubation of nuclei at saturated reagent concentration, when the modification of the most accessible tyrosine was essentially complete. This proved that the reagent itself does not cause unfolding which would result in secondary exposure of internal tyrosine residues. Further experiments showed that the result was the same with crude nuclei isolated in different buffers, with nuclei treated with micrococcal nuclease, with isolated chromatin or nucleosomes, in the absence or in the presence of divalent cations, and in NaCl concentrations from 50 to 400 mM. Thus, the nucleosome structure as probed by tyrosine accessibility is not affected by higher order structures of chromatin. We did not explore the effect of very low ionic strength which had previously been shown to affect the accessibility of histone tyrosines in nucleosomes to iodination (Burch & Martinson, 1981). Kinetic experiments showed that under our conditions, the reagent was exhausted after 15 min because of reaction with the solvent. All experiments were therefore done under the same conditions, with 2 mM NBSF for 10 min at 24 °C.

*Stepwise Dissociation of the Nucleosome Complex.* We took advantage of the well-studied stepwise dissociation of the nucleosome complex in increasing salt concentrations to assign the histone tyrosines to specific binding sites. It had been shown that treating nucleosomes, chromatin, or nuclei with 0.6 M NaCl dissociates histone H1, the basic N-terminal tails of the core histones (Walker, 1984), and other DNA-bound proteins such as RNA polymerase and transcription factors (Dignam et al., 1983), without affecting the structure of the nucleosome core complex (Ausio et al., 1989). Raising the NaCl concentration to 1.2 M causes the dissociation of the H2A–H2B dimers, leaving H3 and H4 bound to the DNA (Burton et al., 1978). At NaCl concentrations of 2 M and higher, the H3–H4 tetramer also dissociates from DNA but now reassociates with H2A–H2B dimers to form hexameric and octameric complexes in the absence of DNA (Eickbush & Moudrianakis, 1978). When histone complexes separated from DNA at high salt concentrations are dialyzed to lower salt concentrations, they dissociate into H2A–H2B dimers and H3–H4 tetramers, which seem to be the stable form of histones under physiological conditions. These histone complexes have been shown to form from acid-extracted histones with very high affinities, resulting in complexes with an increased helical content (D'Anna & Isenberg, 1974).

Nuclei and native chromatin or polynucleosomes form extremely viscous gels at salt concentrations above 0.4 M NaCl. In order to maintain uniform reaction conditions, the concentration of DNA has to be kept below 0.5 mg/mL, or the DNA has to be digested with micrococcal nuclease. We first established that the digestion of nuclei with micrococcal nuclease did not influence the results and then performed all experiments with nuclei digested to a point were the average length of the DNA was 600–1200 bp (3–6 nucleosomes), at a nucleic acid concentration below 1 mg/mL.

Figure 1 shows the HPLC profiles of the histones isolated from calf thymus nuclei treated with NBSF in different concentrations of NaCl. The absorbance profile at 214 nm on the left reflects the amount of protein in each peak; the 254-nm profile on the right identifies the modified forms. It is immediately apparent that with increasing NaCl concentrations more modified forms appear in two phases. While the unmodified forms of some histones disappear completely (e.g., H2B), others remain essentially unchanged (e.g., H2A).

At NaCl concentrations up to 0.4 M, the 214-nm profiles show a 70% decrease in H2B with the simultaneous appearance of a new peak (1) between H2A.2 and H2A.1, which has a high absorbance at 254 nm. At the same time, their is a slight decrease in the H3 peaks and the appearance of small new peaks (e.g., 2) which have shifted to the right and have a high absorbance at 254 nm. Similar changes occur in all H3 variants. For clarity, we will concentrate on the results of H3.1, since this variant constitutes ca. 90% of H3 in calf thymus.

At 0.8 M NaCl, the 214-nm profile shows that H4 and H2A are still essentially unaffected while H2B is now reduced to a very low amount and a new peak (3) is present between peak 1 and H2A.1. From the 254-nm profile, it is apparent that this new peak 3 has about twice the absorbance at 254 nm relative to 214 nm as compared to peak 1. This would be expected for proteins with two modified tyrosines. The same 254-nm profile of histones modified in 0.8 M NaCl also shows the appearance of other new peaks with a high absorbance at 254 nm, namely, peak 4 just to the right of H3.1 and peak 5 to the right of peak 2, with the latter having an absorbance ratio of 254/214 expected for a protein with two modified tyrosines.

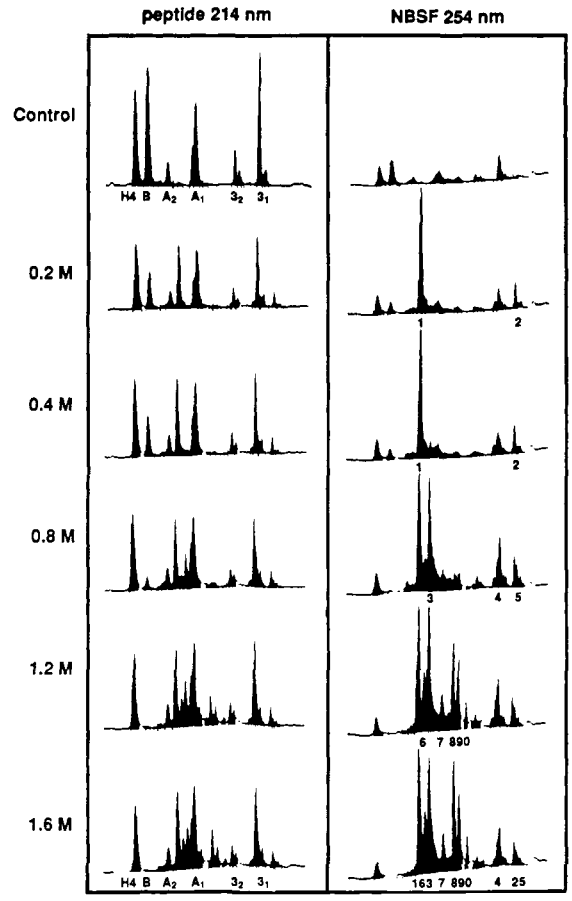


FIGURE 1: NBSF modification of histones at different NaCl concentrations. Histones extracted from calf thymus nuclear homogenates untreated (control) or treated in different NaCl concentrations (0.2–1.6 M) with 2 mM NBSF for 10 min at 24 °C were resolved by reversed-phase HPLC as described under Materials and Methods. The unmodified histones are B = H2B.1, $A_2$ = H2A.2, $A_1$ = H2A.1, $3_2$ = H3.2, and $3_1$ = H3.1 [histone variant nomenclature according to Franklin and Zweidler (1976)]. The eluate was monitored at 214 nm for protein quantitation and at 254 nm for identification of NBSF-modified forms, which are labeled 1 through 0 in order of appearance with increasing salt concentrations.

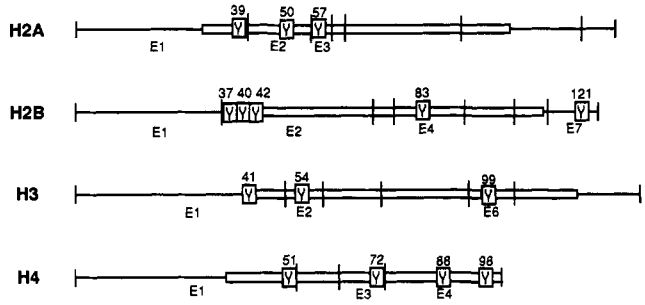


FIGURE 2: Position of the tyrosines in the core histones and their separation into different peptides produced by cleavage with endoprotease Glu-C (E1–E7). For histone amino acid sequences, see van Holde (1988).

At 1.2 M NaCl, the 214-nm profile shows a slight decrease of unmodified H4 relative to H2A and the complete absence of unmodified H2B. At the same time, new peaks appear between peaks 1 and 3 (peak 6), and to the right of H2A.1 (peaks 7, 8, 9, and 0). No further changes are seen in the H3 region. The same pattern is seen in higher concentrations of NaCl.

To identify which tyrosine residues were modified, all the protein peaks with high absorbance at 254 nm were collected and digested with endoprotease Glu-C, which cleaves the histones in such a way that almost all the tyrosine residues

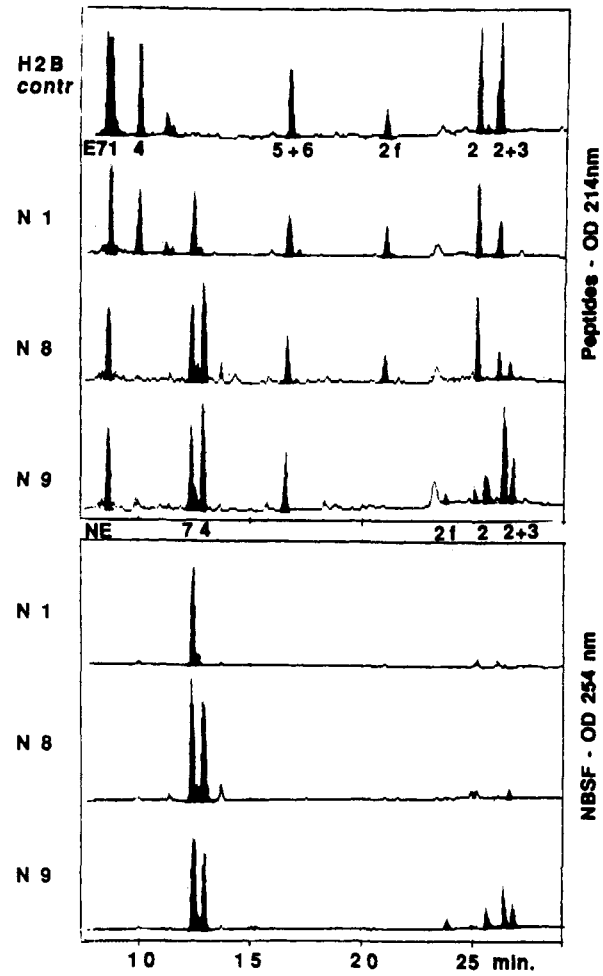


FIGURE 3: Identification of the individual modified tyrosines. Modified proteins 1, 8, and 9 isolated by HPLC as shown in Figure 1 were digested with endoproteinase Glu-C, and the peptides were separated by reversed-phase HPLC. The eluate was monitored at 214 nm to detect all peptides (E1–7) and at 254 nm to identify the NBSF-modified peptides (NE1–7). See Figure 2 for predicted tyrosine-containing peptides of the core histones. E2f is a fragment of peptide E2. Cleavage at H2B–Glu71 and Glu105 is very slow, resulting in partial cleavage products E2+3 and E5+6.

are in different peptides (Figure 2). Only the closely spaced Tyr37, -40, and -42 of H2B and Tyr88 and -98 of H4 are not resolved into separate Glu-peptides. The peptides were then resolved by reversed-phase HPLC. Figure 3 shows the peptide maps of peaks 1, 8, and 9 which on the basis of the peptide pattern are identified as H2B proteins. The 214-nm profile for peak 1 shows the disappearance of peptide E7 and the appearance of a new peptide which elutes ca. 3 min later and has a high absorbance at 254 nm. Amino acid composition confirms this modified peptide as E7 and the modified tyrosine as H2B–Y121. In the 214-nm profile of peak 8, peptides E7 and E4 are missing, and two new peaks with high 254-nm absorbance elute ca. 3 min later. Amino acid composition confirmed the modified peaks as E7 and E4, the latter containing H2B–Y83. The 214-nm peptide map for peak 9 shows in addition to E7 and E4 a shift in E2, and we confirmed the modification of one of the tyrosines in the group H2B–Y37, H2B–Y40, and H2B–Y42. The modified peptides of all other peaks with high absorbance at 254 nm were identified in the same fashion. The results are summarized in Table I.

The assignment of all the modified protein peaks to specific tyrosine modifications allows the quantitation of the extent of modification for each tyrosine directly from the 214-nm absorbance profile of the original chromatogram of total

Table I: Identification of the NBSF-Modified Proteins in Figure 1

| peak (see Figure 1) | histone modified | Glu-peptides (see Figure 2) | Tyr modified |
|---|---|---|---|
| 1 | H2B | E7 | Tyr121 |
| 2 | H3 | E2 | Tyr54 |
| 3 | H2B | E2 + E7 | Tyr37, -40, or -42 + Tyr121 |
| 4 | H3 | E1 | Tyr41 |
| 5 | H3 | E1, E2 | Tyr41 + Tyr54 |
| 6 | H4 | E4 | Tyr88 or -98 |
| 7 | H4 | E3 | Tyr72 |
| 8 | H2B | E4 + E7 | Tyr83 + Tyr121 |
| 9 | H2B | E2 + E4 + E7 | Tyr37, -40, or -42 + Tyr83 + Tyr121 |
| 0 | H4 | E3 + E4 | Tyr72 + Tyr88 or -98 |

histone preparations. Figure 4 shows the changes in the extent of modification of individual tyrosine residues with increasing NaCl concentrations as calculated from the profiles shown in Figure 1 and additional data from the same experiment.

One can distinguish three different patterns of histone tyrosine exposure in nucleosomes at different salt concentrations.

(*a*) *Tyrosines Exposed in Native Chromatin of Intact Nuclei.* H2B–Y121, located near the C-terminus, is the most accessible tyrosine in the nucleosome. It is highly reactive even in condensed chromatin. The extent of modification under our standard conditions increased slightly from ca. 70% in compact lymphocyte chromatin at physiological NaCl concentrations to 100% in completely solubilized chromatin at 1 M NaCl. This increase could be due to a general increase in nucleosome accessibility rather than a specific change in the environment of this tyrosine. Alternatively, the gradual increase in exposure with increased [NaCl] may reflect a weak interaction of H2B–Y121 with the DNA in conjunction with the neighboring lysine residues. H3–Y54, located in a loop within the structured region (Arents et al., 1991), is also accessible at all salt concentrations, but the extent of modification is less than 20% that of H2B–Y121, indicating a more restrictive environment which is not changed by dissociation of the nucleosome complex.

(*b*) *Tyrosines Exposed by Intermediate Salt Concentrations When H1 and the Basic N-Terminal Tails of the Core Histones Are Dissociated from DNA, but the Nucleosome Core Complex Remains Intact.* H2B–Y37, H2B–Y40, or H2B–Y42 and H3–Y41 become increasingly accessible between 0.2 and 0.8 M NaCl. These residues are located next to the very basic N-terminal tails. They most likely are involved in histone–DNA interactions, either directly or indirectly.

(*c*) *Tyrosines Exposed When the H2A–H2B Dimers Dissociate from the Chromatin at 1.2 M NaCl (Burton et al., 1978).* H2B–Y83, H4–Y72, and H4–Y88,98 are located in the structured region of the respective proteins which have been proposed to be involved in protein–protein interactions. These tyrosine residues are therefore most likely in the interaction between the H2A–H2B dimers and the H3–H4 tetramer.

No additional modified forms are found at higher salt concentrations when the H3–H4 tetramers dissociate from the DNA as well. However, the reactivities of the H4-tyrosines at the dimer–tetramer interface tend to decrease again as shown in Figure 3, as expected for the reassociation of H2A–H2B dimers and H3–H4 tetramers at very high salt concentrations in the absence of DNA (Eickbush & Moudrianakis, 1978).

The different maximal rates of reaction of the different tyrosine residues under our standard conditions probably reflect the immediate environment. The most reactive H2B–

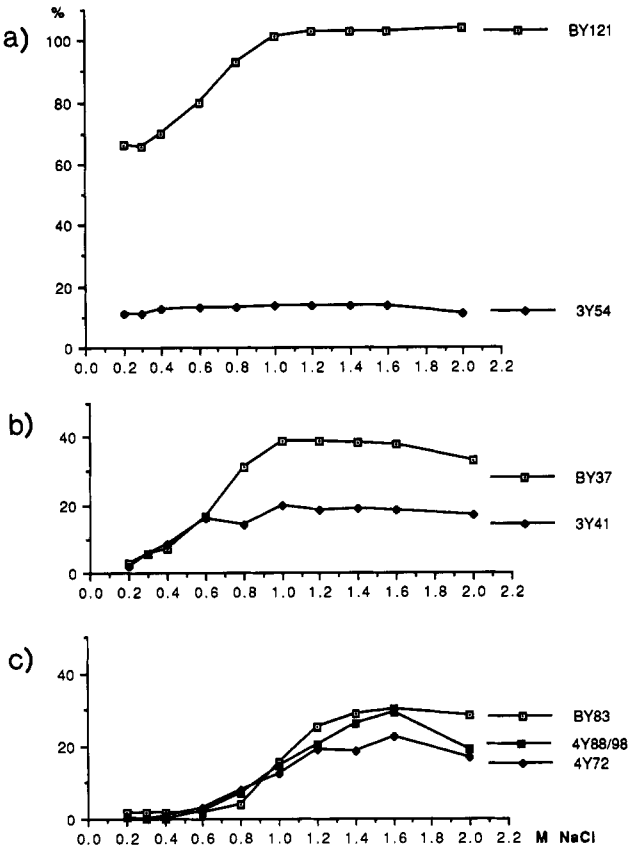


FIGURE 4: Salt concentration dependence of NBSF modification of individual histone tyrosines. The total degree of modification of individual tyrosine residues was calculated from the peak areas in Figure 1 based on the modified forms as shown in Table I. H2B–Y121 (BY121) and H3–Y54 (3Y54) are the only histone tyrosines modified in physiolgical salt concentrations. H2B–37, –40, or –42 (BY37) and H3–Y41 (3Y41) become accessible between 0.2 and 0.8 M NaCl. H2B–Y83 (BY83), H4–Y72 (4Y72), and H4–Y88/98 (4Y88/98) only become accessible between 0.8 and 1.2 M NaCl. The extent of modification of H4–Y88/98 and H4Y72 tends to decrease again between 1.6 and 2 M NaCl, probably reflecting the re-formation of the octameric histone complexes in the absence of DNA.

Y121 is surrounded by small hydrophilic residues in the sequence TKYTSS. The least reactive H3–Y41 and H3–Y54 are surrounded by bulky positively charged residues, HRYRP and RRYQK, respectively, at the beginning of $\alpha$-helices. The tyrosines with intermediate reactivities, H2B–Y83, H4–Y72, and H4–Y88, are all located in $\alpha$-helices.

*Isolated Histone Complexes.* As expected, the removal of DNA did not have any influence on the reactivity of the histone tyrosine residues in histone complexes at 2 M NaCl (data not shown). When the histone octamers separated from DNA in high salt are dialyzed to lower ionic strength, they dissociate into dimers and tetramers. In such isolated "native" histone complexes at physiolgoical ionic strength, the same tyrosines were accessible as in chromatin in 1.4 M NaCl, although there were some differences in the relative amounts of the various modified forms.

The tyrosine residues which remain unreactive in isolated native H2A–H2B dimers (H2A–Y39 –Y50, and –Y57) as well as the H3–H4 tetramers (H3–Y99 and H4–Y51) can be modified when the individual histones are isolated. Histones have to be at least partially denatured to separate the individual components of the naturally occurring complexes. On the other hand, the secondary structure of individual histones does not re-form completely until they are associated in H2A–H2B heterodimers or H3–H4 tetramers (D'Anna & Isenberg, 1974). Others have shown that all the tyrosines can be

iodinated in isolated H2A (Callaway et al., 1985) but are protected in reconstituted H2A–H2B dimers. We found that in acid-extracted individual histones, H4–Y51 and H3–Y99 are modified by NBSF (data not shown).

## DISCUSSION

There is little specific information available about the structure of the histone proteins in the nucleosome complex. X-ray crystallography of nucleosomes has not yet provided data of sufficient resolution to trace the polypeptide chains and to identify the positions of individual residues (Richmond et al., 1984). The only crystal structure of DNA-free histone complexes is of the histone octamer formed at high salt concentrations (Arents et al., 1991). The most recent model of the octamer establishes the three-dimensional arrangement of the structured regions of the histone polypeptides, without details on the position of the side chains. The overall shape of this octamer fits well within the space proposed to be occupied by the histones in the nucleosome crystals.

Our results on the location of histone tyrosine residues at specific interfaces within the nucleosome are entirely consistent with the proposed structure for the DNA-free histone octamer (Figure 5). Specifically, H2B–Y121, H2B–37,40,42, H3–Y41, and H3–Y54, which we identified as being exposed and potentially interacting with DNA in nucleosomes, are all at the surface, while H2B–Y83, H4–Y72, and H4Y88/98 are at the dimer–tetramer interfaces in the octamer model (Arendts et al., 1991). H2A–Y50 and -Y57, as well as H3–Y99, which do not react with NBSF in any native histone complex, are located in the long $\alpha$-helices which form the central part of the intimate "handshake" interactions between H2A and H2B as well as between H3 and H4. H2A–Y39 and H4–Y51, which are also inaccessible to NBSF in native histone complexes, are both in helix–loop–helix motifs which flank the central $\alpha$-helix and are well positioned for either intramolecular folding or the formation of the primary histone complexes, between H2A and H2B as well as H3 and H4.

Our results also agree well with and extend previous studies on the role of tyrosines in histone–histone and histone–DNA interactions in the nucleosome. Early studies using tyrosine iodination were largely restricted to histone H4 in chromatin and gave contradictory results (Biroc & Reeder, 1976; Burch & Martinson, 1981; Griffith & Huang, 1979). This was shown to be at least partially due to conformational changes in chromatin induced by very low ionic strength and by shearing (Martinson et al., 1979), conditions commonly used in the preparation of chromatin. This indicates that the H4 tyrosines at the dimer–tetramer interphase are very sensitive to distortions in the overall chromatin structure. In a tyrosine iodination study of isolated H2A and H2B histones, it was found that all three tyrosines of H2A are occluded by its interaction with H2B while none of the H2B tyrosines were affected by H2A–H2B complex formation (Callaway et al., 1985), consistent with our results. UV differential studies of isolated histones and reconstituted complexes (Michalski-Scrive et al., 1982) also indicated that three of the eight tyrosines in H2A and H2B are internalized when the two histones interact. The finding that iodination of tyrosines in H2A, H2B, and H4 does not inhibit reconstitution of nucleosome cores, but may in some cases even form more stable complexes (Kleinschmidt & Martinson, 1984), could indicate that most of the tyrosines are not buried in the protein interior, where spatial constraints are severe, and that some of the tyrosines may be involved in aromatic–aromatic interactions (Burley & Petsko, 1985), rather than hydrogen bonds.

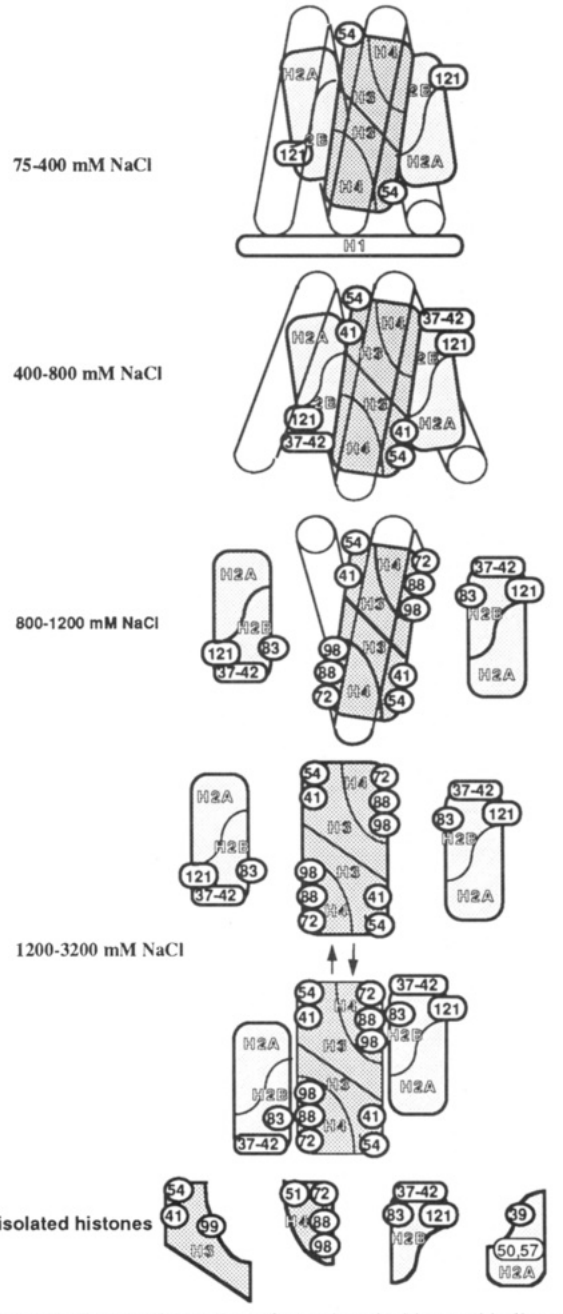


FIGURE 5: Proposed location of tyrosines in histone binding sites within the nucleosome complex. The model is based on the published data on nucleosome structure and the mapping of individual histone tyrosines (identified by their position) at specific binding sites within the nucleosome complex as reported here. The features of the histone complexes are a schematic representation of the complex interactions within the H2A–H2B dimers and the H3–H4 tetramers recently proposed on the basis of the crystal structure of the DNA-free histone octamer (Arents et al., 1991).

Modification of nucleosome core tyrosines with an imidazole spin-label (Chan & Piette, 1982) showed that only 1–2 of the 15 histone tyrosines were accessible from 0.1 to 400 mM NaCl, most likely corresponding to H2B–Y121 and H3–Y54, which we found to be the most accessible tyrosines under these conditions. At high NaCl concentrations, four more tyrosines were accessible to imidazole labeling, possibly including the peripheral H2B–Y39–42 and H3–Y41, but not the H2B and H4 tyrosines at the dimer–tetramer interface which is occluded again in very high salt and protein concentrations. The modification of the "peripheral" tyrosine residues did not interfere with the reconstitution of the nucleosome core particles, while the modification of "internal" tyrosines in the presence of urea did. Tyrosine fluorescence studies (Ashikawa

et al., 1982) also indicated that 6 of the 15 core histone tyrosines were located at the periphery of the histone complex in close proximity with DNA, while the others were internal.

Taken together, the available data indicate that there are 12 histone tyrosine residues per nucleosome core (2 each of H2B–Y37, –Y40, –Y42, and –Y121, H3–Y41, –Y54) which are in a position to interact with DNA. All of these residues are retained in the proteolyzed core particles and could therefore contribute to the stability of the nucleosome complexes after removal of most of the basic residues in the N-terminal regions of the histones (Ausio et al., 1989). The interaction of tyrosine residues can occur by hydrogen bonding either to the phosphate backbone (Jordan & Pabo, 1988) or to the ring nitrogens of the bases (Helene & Maurizot, 1981).

On the basis of the pH sensitivity of the interaction between H2A–H2B dimers and H3–H4 tetramers to form octamers, it had been postulated that histidine–tyrosine hydrogen bonds were involved (Eickbush & Moudrianakis, 1978; Butler & Olins, 1982; Godfrey et al., 1980). Our study places four core histone tyrosine residues (H2B–Y83, H4–Y72, H4–Y88, and H4–Y98) at this interface. That a significant portion of the dimer–tetramer binding site is supplied by H2B–H4 interactions was suggested both by in vitro binding studies (D'Anna & Isenberg, 1974) and by cross-linking studies (DeLange et al., 1979; Martinson et al., 1979). On the basis of our results and the proposed crystal structure for the histone octamer, we propose that H2B–Y83 may form a hydrogen bond with H4–His75 and that either H4–Y72 or H4–Y88 forms a hydrogen bond with H2B–His82.

Our results emphasize the importance of tyrosine residues in all sites of molecular interaction which form the nucleosome complex. The exposure of certain histone tyrosines reflects the occupancy of specific binding sites within the nucleosome complex. Reaction with NBSF provides a simple assay for the accessibility of tyrosine residues and therefore the status of the specific binding sites they are involved in. Thus, the accessibility to NBSF modification of H2B–Y37, H2B–Y40, and H2B–Y42 is a good indicator of the interaction between H2A–H2B dimers and DNA, while H3–Y41 similarly is a good indicator of the H3–H4 tetramer interaction with DNA. The exposure to NBSF modification of H2B–Y83 as well as H4–Y72 and H4–Y88 is a sensitive measure of dimer–tetramer interactions. The reaction of tyrosine with NBSF occurs rapidly under physiological conditions in complex environments. It could therefore provide a valuable tool to test several hypothesis about the mechanisms of chromatin transcription and replication which propose different schemes of nucleosome dissociation [reviewed by van Holde et al. (1992), Jackson (1990); Nacheva et al. (1989), and Morse (1992)].

Tyrosine residues are often located at critical places in polypeptides, especially at sites of interaction between different domains of the same protein or between different proteins. Mapping the accessibility of individual tyrosines by the method described in this report should be a valuable general technique for studying the structure and function of proteins which interact with other macromolecules as part of their function. The NBSF footprinting technique appears to be unusually useful because of the high contrast in reaction rates between surface tyrosines in unoccupied and occupied binding sites, as demonstrated above.

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