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Core Histones Are Glutaminyl Substrates for Tissue Transglutaminase*

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Chicken erythrocyte core histones are glutaminyl substrates in the transglutaminase (TGase) reaction with monodansylcadaverine (DNC) as donor amine. The modification is very fast when compared with that of many native substrates of TGase. Out of the 18 glutamines of the four histones, nine (namely glutamine 95 of H2B; glutamines 5, 19, and 125 of H3; glutamines 27 and 93 of H4; and glutamines 24, 104, and 112 of H2A) are the amine acceptors in free histones. The use of Gln^{112} of H2A requires a temperature-dependent partial unfolding of the histone, showing that structural determinants are decisive for the glutamine specificity. The structures of H2A and H2B do not appreciably change upon modification with DNC as determined by circular dichroism, and core particles reconstituted from these DNC-modified histones are indistinguishable from native nucleosome cores. When the reaction is carried out with native nucleosomes, only glutamines 5 and 19 of H3, which are located in the N-terminal tail, and glutamine 22 of H2B, which is not labeled in free histone, are modified. Methylamine and putrescine also are incorporated into nucleosomes by the TGase reaction. Our results reveal several possibilities for the application of the TGase reaction in the chromatin field, and taking into account that histones are easily cross-linked or modified by polyamines *in vitro*, the possibility that they may be TGase substrates *in vivo* is discussed.

The basic repeating subunit of chromatin, the nucleosome core, contains a histone octamer, formed by two copies each of histones H2A, H2B, H3, and H4, wrapped by 146 base pairs of DNA. The linker histone H1 (as well as H5 in nucleate erythrocytes) interacts with 20 additional base pairs of DNA (for review, see Ref. 1). The path of DNA around the histone octamer has been described (2, 3), but the information on the architecture of the histones in the nucleosome mainly depends on the data obtained from crystals of histone octamers (3, 4). The structures of core histones within the nucleosome and free in solution are similar in that the N-terminal third of the molecules does not possess any regular structure, whereas the remaining two-thirds adopt a compact structure. The C-terminal tract of H2A may be an exception in that it seems to expand radially from a central position within the core to the DNA surface at the dyad axis (3). The flexibility of the N-terminal tails makes it difficult to know in detail their paths in the nucleosome, and yet the issue is an interesting one, for these tails contain the biologically acetylatable lysyl residues (see Ref. 5 for a recent review on histone acetylation).

The introduction of reporter groups (for instance, fluorescent probes) into proteins has often be a valuable experimental approach to study structural details either in the proteins themselves or in supramolecular complexes involving these proteins. However, the use of fluorescent probes in histones and nucleosomes has been very limited to date because of the difficulty in obtaining specifically modified histones. One remarkable exception is the covalent modification of cysteines by 5-[(iodoacetamido)ethyl]aminonaphthalene-1-sulfonic acid, but its use is obviously limited to cysteine-containing histones, *i.e.* H3 of most organisms (reviewed in Ref. 6) and H4 in some cases (7). Of course, the specific modification of a residue more abundant in core histones might overcome that difficulty.

Enzymatically directed chemical modification of proteins is arousing considerable interest, and for instance, the reaction catalyzed by transglutaminase (TGase)[1] has been used to introduce fluorescent probes into proteins or peptides. The fluorescent derivatives have proved to be useful and sensitive tools to study the interactions between the peptides or proteins and their physiological counterparts in complex systems (see, for instance, Ref. 8).

TGases catalyze an acyl transfer reaction in which the γ-carboxamide group of a glutamine residue in a protein acts as acyl donor. The ϵ-amino group of lysine residues as well as some polyamines are the physiological amine donors, but some non-physiological amines can also be used by the enzyme (9). The reasons for the specificity of protein lysyl or glutaminyl substrates are not known. It seems clear that both the sequence around a particular glutamine residue and the conformation of its neighborhood play a definite role in determining its capacity as substrate (10, 11), and the nature of the amino acid preceding the lysyl substrates is also crucial (12). TGases are involved in a variety of roles, including blood clotting and coagulation of other body fluids, stabilization of intra- and extracellular matrices, formation of the cornified envelope and other related changes in the epidermis, and cross-linking of cell envelopes in apoptosis (10).

In this paper, we report that core histones are remarkably good glutaminyl substrates for tissue TGase. They incorporate DNC faster than many physiological TGase substrates, although, to date, no role for the TGase reaction has been described in histones. We have determined the glutamine resi-

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[1] The abbreviations used are: TGase, transglutaminase; DNC, monodansylcadaverine; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)-ethanesulfonic acid; AUT, acetic acid/urea/Triton X-100; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

dues that act as acyl donors in each of the four individual histones. This basal reactivity changes when histones are incorporated in nucleosomes, and so our results open up a number of possibilities to address structural issues in nucleosomes. Some of the fluorescent histones obtained may represent useful tools to study the histone anatomy in the core particle as well as some of the conformational changes undergone by the nucleosome.

EXPERIMENTAL PROCEDURES

Biochemicals—Guinea pig liver TGase (EC 2.3.2.13), trypsin (EC 3.4.21.4) treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, and DNC were from Sigma. Micrococcal nuclease (EC 3.1.31.1) was purchased from Boehringer Mannheim. Solvents for HPLC were from Merck. [^{14}C]Methylamine hydrochloride (57 mCi/mmol) and [^{14}C]putrescine dihydrochloride (109 mCi/mmol) were purchased from Amersham International. All other chemicals were analytical grade.

Preparation of Nucleosomes and Histones—Chicken erythrocyte nuclei were obtained as described by Weintraub *et al.* (13). Nucleosome cores were prepared from H1/H5-depleted chromatin (14), and DNA was obtained from nucleosome cores by standard procedures. Individual histone fractions were prepared from chicken erythrocyte nuclei (15), and they were further purified by reversed-phase HPLC on a Delta-Pak C_{18} column (Waters) eluted with an acetonitrile gradient (20–60%) in 0.3% trifluoroacetic acid (16). The purity of histones was routinely checked by PAGE (see below).

Incorporation of DNC into Histones or Nucleosomes—The standard incubation mixture contained purified individual histones (1 mg/ml), 12 mM DNC, 40 mM $CaCl_2$, 20 mM dithiothreitol, 600 mM NaCl, guinea pig liver TGase (0.25 units/ml, unless otherwise stated), and 100 mM MES, pH 6.0. The mixture was incubated at either 4 or 37 °C for the times indicated in each experiment. Reactions were stopped by adding trifluoroacetic acid to a final concentration of 1% (v/v), and the excess free unreacted DNC was removed either with a Sephadex G-25 (PD-10) column or by dialysis. When the reaction products were to be analyzed only by PAGE, the reaction was stopped by adding the corresponding sample solvent at an appropriate concentration.

The incorporation of the fluorescent probe into nucleosome-bound histones was carried out by incubating nucleosome cores (10–20 A_{260} units/ml) with 9 mM DNC, 4.5 mM $CaCl_2$, 20 mM dithiothreitol, 10 mM NaCl, 0.5 units/ml guinea pig liver TGase, and 40 mM Tris-HCl buffer, pH 7.4. Incubation was carried out at 30 °C for 1 h, and the reaction was stopped by adding H_2SO_4 to a final concentration of 0.1 M. The samples were stirred for 4 h at 4 °C, and they were then centrifuged to remove the precipitated material. Histones were recovered from the supernatant by standard procedures (acetone precipitation, washing, and drying under vacuum) and processed as described below.

Incorporation of Other Amines into Histones—The incorporation of other amines into histones, either free or in nucleosomes, was tested by using [^{14}C]methylamine and [^{14}C]putrescine. Free histones were labeled with methylamine as described above for DNC, except that the incubation mixture contained 150 mM methylamine (final concentration), with a specific activity of 810 μCi/mmol. To label nucleosome-bound histones, the assay was similar to that performed with DNC, except that the incubation mixture contained either 23 mM methylamine (specific activity of 1.14 mCi/mmol) or 3.1 mM putrescine (specific activity of 49.6 mCi/mmol). The elution of radioactive histones or peptides was monitored with an on-line Beckman 171 radioisotope detector equipped with a liquid detector.

Identification of Glutamine Substrates in Histones—DNC-labeled histones were digested with trypsin at an enzyme/histone ratio of 1:40 (w/w) in 200 mM *N*-ethylmorpholine buffer, pH 8.1. Hydrolysis was carried out at 37 °C for 4 h. The reaction was stopped by placing the tubes in boiling water and freeze-drying after removing the excess DNC on PD-10 columns. When trypsinization was carried out with histones modified in nucleosomes, the H_2SO_4-soluble histone mixture was first resolved into individual histone fractions by HPLC as described above.

Tryptic peptides were separated by reversed-phase HPLC using a Delta-Pak C_{18} column (15 × 0.78 cm). Elution was carried out by either of the two following procedures: procedure A, linear gradient of acetonitrile (0–55%) in 0.1% trifluoroacetic acid (11); and procedure B, linear gradient of propan-2-ol (0–50%) in the presence of 0.4% triethylamine, adjusted to pH 2.5 with H_3PO_4 (17). Fluorescence was monitored (excitation wavelength, 330 nm; and emission wavelength, 510 nm) with an F-1050 fluorescence spectrophotometer (Merck-Hitachi), and the fluorescent peptides were recovered. In some instances, to achieve a complete purification of the peptides, both chromatographic procedures were used.

The amino acid composition of purified fluorescent tryptic peptides was determined, after acid hydrolysis, by HPLC analysis of the phenylthiohydantoin-derivatives (18). Peptides were sequenced, when necessary, in a Model 6625 Prosequencer (Milligen). The phenylthiohydantoin-derivatives resulting from Edman degradation were divided into two aliquots; one of them was analyzed in an on-line HPLC system to identify the amino acid, while the other was saved for fluorescence measurements. The DNC derivative of glutamine, which was not identified in the phenylthiohydantoin analysis, was detected in this way. Cyanogen bromide cleavage of histone H4 was carried out by standard procedures (19), and the resulting peptides were separated by HPLC under the conditions used to purify histones (see above).

Circular Dichroism—The structures of DNC-modified H2A (referred to hereafter as DNC-H2A) and DNC-H2B were studied by circular dichroism. Spectra in the far-UV (195–240 nm) and near-UV (250–300 nm) regions were recorded in a CD-6 dichrograph (Jobin-Yvon) calibrated with isoandrosterone. Spectra were obtained at a protein concentration of ~0.3 mg/ml in cells of 0.1- and 10-mm optical paths for the far- and near-UV regions, respectively. Scan speed was 0.5 nm/s, and the integration constant was set at 1 s. The average of at least three runs was expressed in molar ellipticities with the dimensions of degrees·cm^2·(dmol of residue)$^{-1}$ in the far-UV region or degrees·cm^2·(dmol of protein)$^{-1}$ in the near-UV region.

Nucleosome Reconstitution and Analysis—Nucleosome cores were reconstituted from either unmodified or DNC-modified core histones and DNA of core size following the procedure of Marvin *et al.* (20). The integrity of reconstituted particles was checked by native gel electrophoresis (21), DNase I digestion (22) and ion-exchange HPLC on a DEAE-5PW column (23).

Electrophoresis of Histones—SDS-PAGE was carried out on 15% gels (24). When fluorescently labeled histones were run, gels were photographed under ultraviolet light with a Polaroid MP-4 camera equipped with a Kodak No. 22 Wratten filter, prior to Coomassie Blue staining, to locate the fluorescent histones. When required, Coomassie Blue-stained gels and the negatives of fluorescence images were scanned to estimate the amount of DNC incorporated into individual histones. In some instances, AUT-PAGE was used (25).

RESULTS

Incorporation of DNC into Free Core Histones—Individual histones were incubated with DNC under standard conditions (see above), and the reaction products were resolved by PAGE in the presence of SDS (Fig. 1). All four core histones incorporate the fluorescent probe, and therefore, they can act as glutaminyl substrates for TGase. The intensity of the fluorescence incorporated into individual histones was normalized relative to the amount of histone in each lane, which was estimated by scanning the Coomassie Blue-stained bands (no corrections for differential staining were made). This gave the following order of preference for the histones acting as substrates for TGase: H3 > H2A > H2B > H4.

In the electrophoresis of Fig. 1, all the wells were loaded with an equivalent amount of histones. Nevertheless, the H4 and, especially, H3 lanes show that the amount of monomeric histones dramatically diminishes during incubation. Concomitantly, an increase in polymerized material that does not enter the gel was observed, especially in H3 at 37 °C. Actually, after 30 min of incubation, the H3 band is hardly detected in the stained gel, and yet its fluorescence is plainly visible, thus giving the previously mentioned high ratio of fluorescence intensity. The increase in polymerization may be due to the well known property of H3 and H4 of forming stable aggregates (26), which may be further stabilized by TGase-catalyzed cross-linking.

In the case of H2A and H2B, cross-linking is not as prominent, although, especially in the H2A lanes, fluorescent bands moving slower than core histones are visible. The possibility that these fluorescent products corresponded to linker histones that might be present as contaminants of the core histones was ruled out by incubating a preparation of chicken erythrocyte

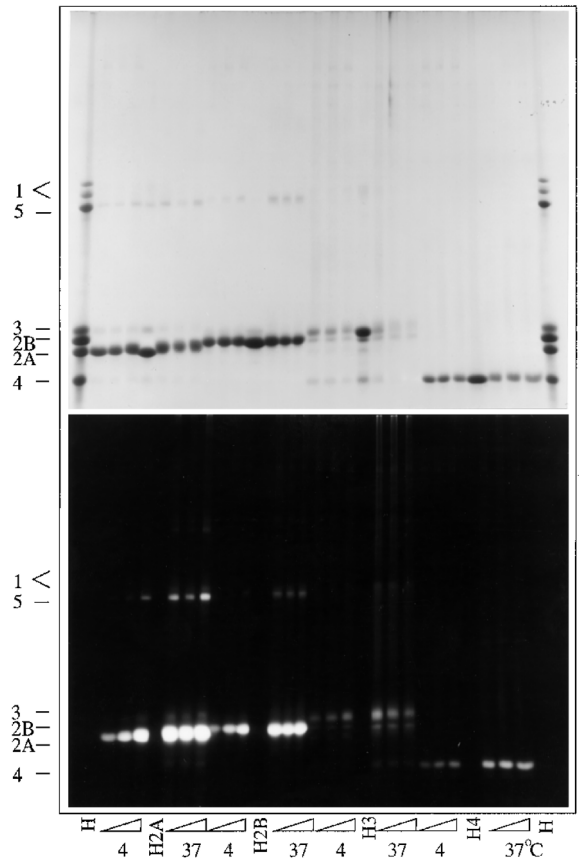

FIG. 1. **Incorporation of the fluorescent probe DNC into the four core histones by the TGase-catalyzed reaction.** Each of the four chicken erythrocyte core histones was incubated with DNC in the presence of TGase, and the products were separated by SDS-PAGE. In the *first* and *last lanes*, unincubated whole histones (*H*) were run as standards. For each individual histone, seven lanes were run: the *center lane*, identified by the histone name, was loaded with unmodified histone as a control, and the *three adjacent lanes* on each side were loaded with histones incubated with DNC in the presence of TGase at 4 or 37 °C, as indicated, for increasing times (15, 30, and 60 min), as denoted by the *triangles*. The *upper panel* shows the Coomassie Blue-stained gel, and the *lower panel* is a photograph of the gel, obtained under ultraviolet light prior to staining, showing the fluorescence of the DNC-modified histones. The mobility of individual unmodified histones is shown on the left.

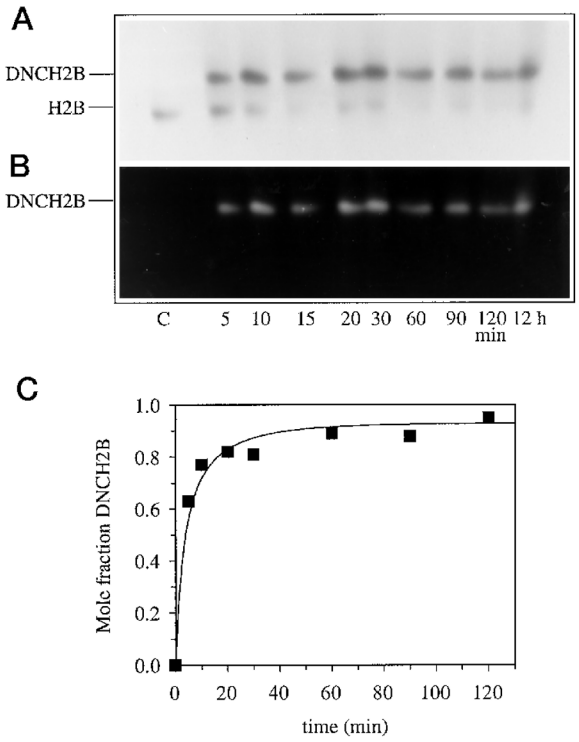

FIG. 2. **Time course of DNC enzymatic incorporation into histone H2B.** H2B was incubated at 37 °C under the conditions described under "Experimental Procedures," and aliquots of the reaction mixture were taken at the indicated times. The reaction was stopped, and the histone was separated from its modified form by AUT-PAGE (25). The gel was stained with Coomassie Blue (*A*) after photography under ultraviolet light (*B*). The *first lane* (control (*C*)) was loaded with unmodified H2B. The stained gel (*A*) was used to obtain, by integrating the bands of unmodified (H2B) and modified (DNC-H2B) histones at each time, the mole fraction of modified H2B, which was plotted against time in *C*.

H1/H5 with DNC under the standard assay conditions. No fluorescent band was detected in these experiments (data not shown), and therefore, the slow moving fluorescent bands appearing in the H2A lanes also correspond to TGase-cross-linked core histones.

DNC Labeling of Histone H2B—AUT-PAGE is able to resolve histones from their DNC derivatives as shown for H2B in Fig. 2*A*. As expected, DNC-H2B moves slower than unmodified H2B, due to an increase in Triton binding when the nonpolar dansyl group is attached to the histone. The fact that a single fluorescent band appeared was a first indication that only a glutamine residue was being labeled under our conditions, as demonstrated below. The good resolution between the H2B and DNC-H2B bands in Fig. 2*A* allowed us to follow, by scanning the gels, the time course of DNC incorporation into H2B, which is given in Fig. 2*B*.

To identify the acyl donor glutamine(s) in H2B, DNC-H2B was digested with trypsin, and the tryptic peptides were separated by HPLC (Fig. 3). A single symmetrical fluorescent peptide was obtained (Fig. 3, *center panel*), and the peak in the corresponding absorbance profile (Fig. 3, *upper panel*) also was symmetrical and well resolved from the neighbor peptides. Therefore, the fluorescent peptide, designated as p1(H2B), was recovered and analyzed without additional purification. When its amino acid composition (Table I) was compared with those of the theoretical tryptic peptides of the histone, it was obvious that p1(H2B) corresponded to peptide 93–99 (theoretical composition of AEIQRTV). This peptide contains a single glutamine residue, Gln^{95}, which was thus identified as the glutaminyl substrate for TGase in H2B.

DNC Labeling of Histone H2A—The incorporation of DNC into H2A is a complex process, as revealed by AUT-PAGE. When incubation was carried out at 37 °C, up to three major fluorescent bands could be detected in the electrophoresis, suggesting that more than one glutamine residue was labeled with the fluorescent probe, but incubating H2A under milder conditions (0.12 units/ml TGase at 4 °C for 30 min) gave simpler patterns on AUT-PAGE (data not shown). The virtual absence of histone cross-linking at 4 °C (Fig. 1) made the initial analysis easier. The HPLC separation of the tryptic peptides by procedure A (Fig. 4) gave two major fluorescent peaks with retention times of 32 and 38 min, designated as p1(H2A) and p2(H2A), respectively. The composition of p1(H2A) (Table I) clearly corresponds to that of peptide 21–29 of the histone (theoretical composition of AFG_2LPQRV), which contains a single Gln residue. This allowed us to identify Gln^{24} as a substrate for TGase. The composition of peptide p2(H2A) (Table I) is very close to that of peptide 100–118 ($A_2DGIKL_3P_2Q_2TV_3$), which contains two glutamines. The sequence of p2(H2A) was determined with the following result: *X*TIA□GGVLPNIQAVLLPK. Although the first amino acid could not be identified, the sequence matched that of peptide 100–118, as anticipated by the amino

acid analysis. In the above sequence, □ means that no conventional phenylthiohydantoin was obtained in cycle 5. According to Murthy *et al.* (27), this result and the resuming of the sequence in cycle 6 indicate that the modified glutamine was present in cycle 5. This interpretation was reinforced by the fact that a clear glutamine signal was obtained in cycle 13. Therefore, Gln^{104} can be identified as the other major glutaminyl substrate of TGase in H2A. Increasing the incubation time at 4 °C to >30 min did not originate additional fluorescent peptides that could indicate the use of a third glutamine as substrate.

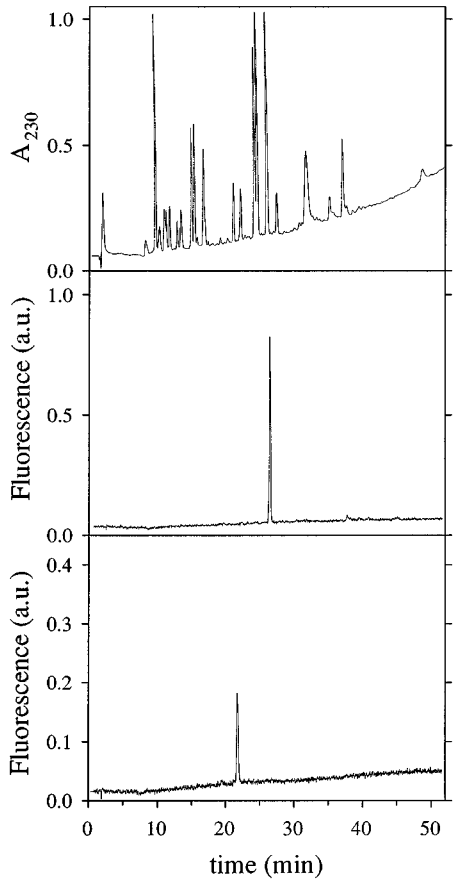

FIG. 3. **Tryptic cleavage of DNC-modified H2B.** Free histone H2B was enzymatically modified with DNC for 60 min under the conditions used in the experiments of Fig. 2. DNC-H2B was then digested with trypsin, and the tryptic peptides were resolved by reversed-phase HPLC. The absorbance (230 nm) (*upper panel*) and fluorescence (excitation at 340 nm and emission at 510 nm) (*center panel*) profiles are shown. The *lower panel* shows the fluorescence profile of a tryptic digest of DNC-H2B obtained by incubating chicken erythrocyte nucleosomes with DNC at 30 °C for 1 h. Note that the peptides labeled in both experiments are different. *a.u.*, arbitrary units.

When TGase-directed incorporation of DNC took place at 37 °C for 90 min, peptide p1(H2A) increased, peptide p2(H2A) decreased, and a novel fluorescent peptide with a retention time of 42 min, designated as p3(H2A), appeared (chromatogram not shown). Amino acid analysis of the latter peptide (Table I) gave a composition similar to that of peptide 100–118. This fact, together with the decrease in p2(H2A) and the concomitant appearance of p3(H2A), allowed us to conclude that p3(H2A) actually corresponds to H2A tryptic peptide 100–118, with both of its glutamines, Gln^{104} and Gln^{112}, modified. Therefore, for Gln^{112} to be a substrate for TGase, a certain temperature is needed.

Glutamine Substrates in H3 and H4—H3 was incubated under standard conditions for 2 h at 37 °C, and tryptic peptides were resolved by HPLC procedure B. Four major fluorescent peptides were recovered and purified by HPLC procedure A. The peptides, designated as p1(H3), p2(H3), p3(H3), and p4(H3), were analyzed for their amino acid composition (Table I). By comparing the figures in Table I with those derived from the composition of the theoretical H3 tryptic peptides, it seems obvious that peptide p4(H3) corresponds to peptide 123–128 (DIQLAR), which contains Gln^{125}, and that peptide p1(H3) corresponds to the peptide containing Gln^{5} (QTAR). The identification of both peptides was unambiguously confirmed by sequencing (data not shown). The composition of peptides p2(H3) and p3(H3) does not match any of the theoretical tryptic peptides. The sequence of p2(H3) is K□LATK. Fluorescence characteristic of the dansyl group was found in the second Edman cycle, so it can be confidently concluded that peptide p2(H3) corresponds to peptide 18–23 (KQLATK), containing Gln^{19}. Finally, peptide p3(H3) gave the sequence □LATK, and dansyl fluorescence was found in the first Edman cycle. Therefore, Gln^{19} appeared in peptides p2(H3) and p3(H3), the former resulting from a partial tryptic hydrolysis.

Chicken H4 contains only two glutamines, Gln^{27} and Gln^{93}, and a single methionine, Met^{84}. To determine the glutamine(s) used by TGase, we cleaved the histone labeled with DNC at 37 °C for 1 h under standard conditions with cyanogen bromide

TABLE I
Amino acid composition of tryptic peptides containing the modified glutamine residues
The amino acid composition is given as number of residues relative to Glu (one or two Glu residues, depending on the peptide).

Amino acid	Peptide								
	p1 (H2B)	p2 (H2B)	p1 (H2A)	p2 (H2A)	p3 (H2A)	p1 (H3)	p2 (H3)	p3 (H3)	p4 (H3)
Asp	—[a]	—	0.2	0.9	1.0	—	—	—	0.8
Glu	2.0	1.0	1.0	2.0	2.0	1.0	1.0	1.0	1.0
Ser	—	—	—	0.2	—	—	0.2	0.1	—
Gly	—	—	2.2	2.3	2.1	—	0.4	0.1	—
His	—	—	—	—	—	—	—	—	—
Thr	1.3	0.8	—	0.9	0.9	1.0	0.8	0.9	—
Ala	1.4	—	1.4	2.5	2.2	1.3	1.3	1.3	1.0
Pro	—	—	1.1	2.4	1.6	—	—	—	—
Arg	1.1	—	1.7	—	—	1.0	—	1.4	0.7
Tyr	—	—	—	—	—	—	—	—	—
Val	1.0	—	1.1	2.9	2.6	—	—	—	—
Met	—	—	—	—	—	—	—	—	—
Ile	0.8	—	—	2.1	3.4[b]	—	—	—	0.7
Leu	1.3	—	1.5	3.2	—	—	0.8	—	0.7
Phe	—	—	0.9	—	—	—	—	—	—
Lys	—	1.1	—	1.1	1.0	—	2.1	1.0	—

[a] —, the amino acid was absent or below the integration range.
[b] The peaks of leucine and isoleucine were not resolved in this experiment. The figure given represents the sum of both amino acids.

and resolved the two peptides by HPLC. Fluorescence was found in both peptides at roughly equivalent intensity (data not shown), and therefore, both glutamine residues act as amine acceptors.

To determine the order of preference among all the different glutamines hitherto mentioned, we combined the data of Fig. 1 (at 37 °C) with those on the relative usage of every glutamine from each single histone. The latter were obtained by integrating the areas of the HPLC-resolved peptides from every single histone, and the results are given in Table II.

Nucleosomes as Substrates for TGase Reaction—Chicken erythrocyte nucleosomes were labeled with DNC, and the histones were isolated and separated by HPLC. Dansyl fluorescence was present only in H2B and H3, thus indicating that, although all four free core histones are TGase substrates *in vitro*, H2A and H4 are not modified in nucleosomes. A single fluorescent peptide appeared in the tryptic digest of H2B, which, surprisingly enough, was not p1(H2B), but rather a novel peptide with a retention time of 21 min (Fig. 3, *lower panel*), designated as p2(H2B), whose composition (Table I) corresponds to that of peptide 21–23 (theoretical composition of KQT), containing Gln^{22}. Interestingly enough, this glutaminyl residue is not modified in the free histone (see Fig. 3, *center panel*). The possible reasons for this behavior will be discussed below. The comparison of the fluorescent tryptic peptides from DNC-H3 isolated from nucleosomes with those obtained from DNC-labeled free H3 allowed us to conclude that two glutamines, namely Gln^5 and Gln^{19}, are the major substrates in nucleosome-bound H3.

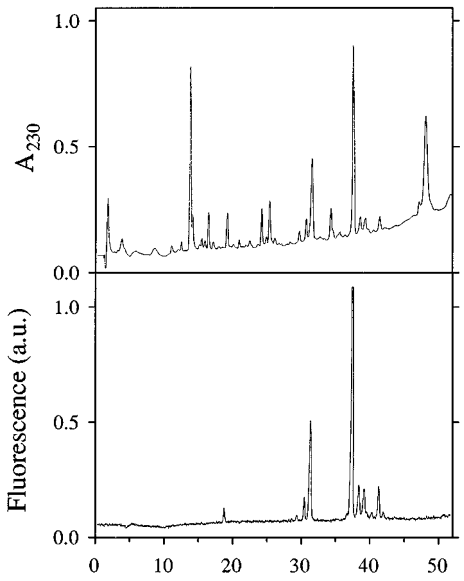

FIG. 4. **Tryptic cleavage of DNC-modified H2A.** Free histone H2A was enzymatically modified with DNC under mild conditions (30 min at 4 °C). DNC-H2A was then digested with trypsin, and the tryptic peptides were resolved by reversed-phase HPLC. The absorbance (*upper panel*) and fluorescence (*lower panel*) profiles are shown. All the chromatographic conditions are as described for Fig. 3. *a.u.*, arbitrary units.

On the other hand, nucleosomes effectively incorporate amines such as putrescine and methylamine into H2B and H3. Although glutamine usage was not determined, it is highly probable that the glutaminyl substrates were the same used by DNC. At least, it was found that free H2B incorporates [^{14}C] methylamine in a single peptide, whose retention time is compatible with its being peptide 93–99 (data not shown).

Circular Dichroism of DNC-labeled Histones—The changes in circular dichroism of DNC-H2B containing Gln^{95} and DNC-H2A labeled in Gln^{24} and Gln^{104} in going from low to high ionic strength are depicted in Fig. 5, compared with those of the unmodified individual histones. In both cases, the ionic strength-induced conformation of the DNC-modified histones is quite similar (as far as the circular dichroism experiments are able to show) to that of the control histones, although the mechanisms of folding in DNC-H2B may be different from those followed by the unmodified histone (see Fig. 5*B*, *inset*). Spectra in the near-UV region (not shown) confirmed the above conclusion.

Nucleosome Reconstitution with DNC-labeled Histones—Due to the likeness of the structures of unmodified histones and their DNC derivatives, we wondered whether the latter could be used to reconstitute nucleosomes. Fig. 6 shows that core particles reconstituted from histone mixtures containing DNC-H2A, DNC-H2B, or DNC-H3 were indistinguishable from those reconstituted from unmodified histones in electrophoretic mobility on native gels and DNase I digestion pattern. The data of Fig. 6*A*, together with those from other similar experiments, indicate that the yield of nucleosome reconstitution from unmodified histones ranks from 55 to 70%, whereas the yield in

TABLE II
Usage of glutaminyl residues of histones as TGase substrates

The glutamine residues identified as TGase substrates in free histones are listed, including a semiquantitative estimation of their substrate capability, ranked from +++ for the most effective substrate to ± for the glutamines modified at a lower rate. The sign − means that these glutamines were not appreciably modified after 1 h of incubation at 37 °C.

Residue	Relative usage	Sequence around the residue										
		−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5
Gln^5 (H3)	+++		A	R	T	K	Q	T	A	R	K	S
Gln^{19} (H3)	++	K	A	P	R	K	Q	L	A	T	K	A
Gln^{104} (H2A)	++	K	V	T	I	A	Q	G	G	V	L	P
Gln^{95} (H2B)	++	T	S	R	E	I	Q	T	A	V	R	L
Gln^{24} (H2A)	++	S	R	A	G	L	Q	F	P	V	G	R
Gln^{125} (H3)	+	M	P	K	D	I	Q	L	A	R	R	I
Gln^{112} (H2A)	+	V	L	P	N	I	Q	A	V	L	L	P
Gln^{27} (H4)	±	L	R	D	N	I	Q	G	I	T	K	P
Gln^{93} (H4)	±	Y	A	L	K	R	Q	G	R	T	L	Y
Gln^6 (H2A)	−	S	G	R	G	K	Q	G	G	K	A	R
Gln^{84} (H2A)	−	I	P	R	H	L	Q	L	A	I	R	N
Gln^{22} (H2B)	−	A	V	T	K	A	Q	K	K	G	D	K
Gln^{47} (H2B)	−	Y	K	V	L	K	Q	V	H	P	D	T
Gln^{55} (H3)	−	E	I	R	R	Y	Q	K	S	T	E	L
Gln^{68} (H3)	−	R	K	L	P	F	Q	R	L	V	R	E
Gln^{76} (H3)	−	V	R	E	I	A	Q	D	F	K	T	D
Gln^{85} (H3)	−	T	D	L	R	F	Q	S	S	A	V	M
Gln^{93} (H3)	−	A	V	M	A	L	Q	E	A	C	E	A

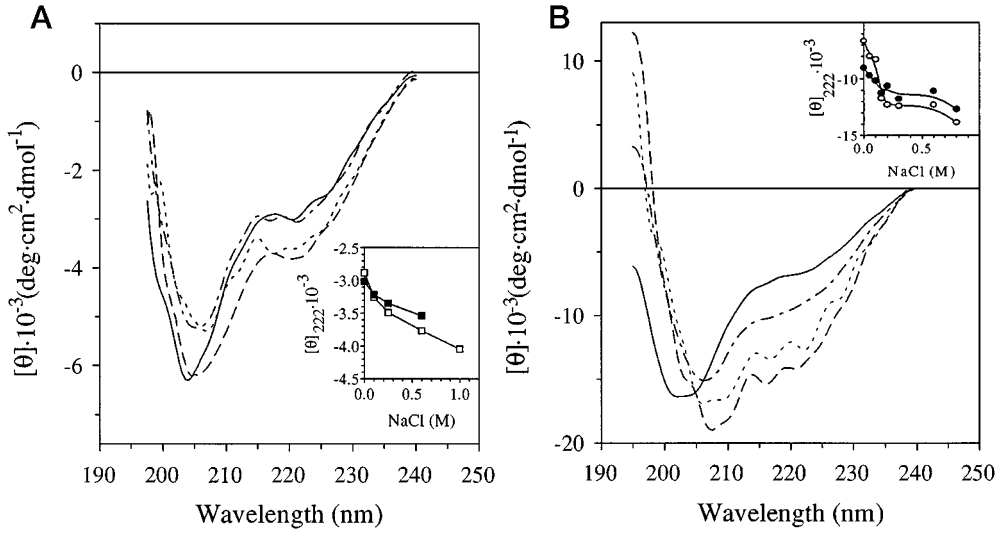

FIG. 5. **Circular dichroism of unmodified and DNC-modified histones.** CD spectra were recorded in the far-UV region at various ionic strengths as described under "Experimental Procedures." *A*, H2A and DNC-H2A; *B*, H2B and DNC-H2B. ——, unmodified histones in 30 mM Tris-HCl, pH 7.0; – – – -, DNC-modified histones in the same buffer; – – –, unmodified histones in Tris buffer containing NaCl (0.6 M in *A* and 0.8 M in *B*); - - - - -, DNC-modified histones in Tris buffer containing NaCl (0.6 M in *A* and 0.8 M in *B*). The *insets* show plots of molar ellipticity at 222 nm against NaCl concentration in Tris buffer. *Open symbols*, unmodified histones; *closed symbols*, DNC-modified histones. *deg*, degrees.

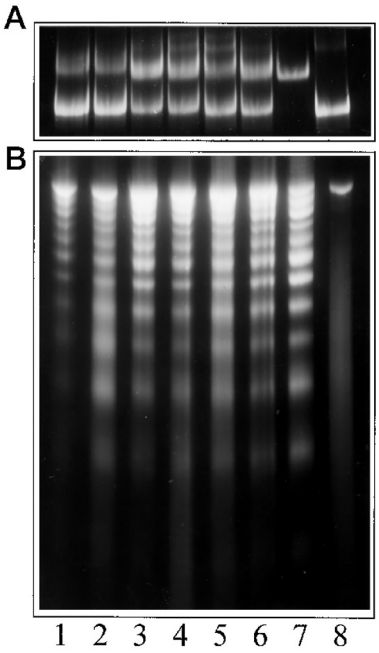

FIG. 6. **Effects of histone dansylation on nucleosome structure.** Nucleosome cores were reconstituted from nucleosomal-size DNA and unmodified or dansylated core histones, and the reconstituted material was analyzed by PAGE under native conditions (*A*) or by denaturing PAGE after DNase I digestion (*B*). *Lanes 1–5* in *A* and *B* were loaded with nucleosomes reconstituted with a mixture of unmodified histones and a single dansylated histone: *lane 1*, DNC-H2A (Gln^{24} and Gln^{104}); *lane 2*, DNC-H2B (Gln^{95}); *lane 3*, DNC-H2B (Gln^{22}); *lane 4*, DNC-H3 (Gln^{5} and Gln^{19}); *lane 5*, DNC-H3 (Gln^{5}, Gln^{19}, and Gln^{125}). *Lane 6* shows the results of reconstitution with all histones unmodified. As controls, *lanes 7* and *8* show the profiles of native nucleosomes and the free DNA used in the reconstitution experiments, respectively. The yields of nucleosome reconstitution in this particular experiment were 25, 30, 60, 50, 35, and 55% for *lanes 1–6*, respectively.

the presence of one DNC-modified histone is 25–60%. In spite of the lower yield, the nucleosome cores reconstituted from DNC-modified histones elute from an ion-exchange HPLC column (see "Experimental Procedures") with the same retention time as native cores or core particles reconstituted from unmodified histones, and the reconstituted cores exhibited DNC fluorescence, showing that DNC-modified histones actually are incorporated into nucleosomes. Moreover, all these nucleosomes gave a similar DNase I ladder (Fig. 6*B*), showing that the DNA path is not altered when DNC-modified histones are used in the reconstitution. The labeling of native nucleosome cores with DNC, which results in the incorporation of the probe in Gln^{5} and Gln^{19} of H3 and Gln^{22} of H2B (see above), also maintains the integrity of the nucleosome (data not shown).

DISCUSSION

In this paper, we show that 9 out of the 18 glutamine residues present in free core histones are glutaminyl substrates for TGase *in vitro*, and we have identified these nine residues. The basis for glutamine specificity in the TGase-catalyzed reaction remains obscure, and in spite of the effort carried out by several authors, it has not been possible to derive a consensus sequence for the glutaminyl substrates (11, 17). Our data show no clear dominance of any kind of amino acids in the environment of the modified glutamines when compared with the unmodified ones (Table II). In close agreement with previous observations made by other workers (10, 11, 17), it can be concluded that the lack of a consensus sequence may reflect an important dependence on the conformation of the glutamine environment.

Our data also support the idea that the conformational determinants are essential for a glutamine residue to be modified because of the temperature-dependent modification of Gln^{112} of H2A. Obviously, apart from sequence motifs, the final reason that determines whether Gln^{112} is modified or not must be a structural one. The structure of histones in solution changes in going from 4 to 37 °C (28), and the modification of Gln^{112} therefore depends on the temperature-related exposure of the residue.

Of the 18 glutamines of core histones (Table II), five are placed in the N-terminal tails. Three of these, namely Gln^{24} of H2A and Gln^{5} and Gln^{19} of H3, are TGase substrates in free histones, whereas Gln^{6} of H2A and Gln^{22} of H2B are not. By studying a number of proteins whose glutamines are known either to act or not to act as TGase substrates, Coussons *et al.* (29) derived some rules to identify "discouraging" sequence features for the TGase reaction. For substrates with little ordered structure, they claimed that the positively charged amino acids within the five positions next to the C-terminal

side of a glutamine residue are a discouraging feature, whereas glycine, valine, and leucine frequently occur on the C-terminal side of the modified glutamines. These rules do not fully apply to the N-terminal tails of the histones. The best glutamine substrate in the histones, namely Gln^5 of H3, possesses two positively charged residues in positions +3 and +4 relative to glutamine, and the other two glutamines modified in the N-terminal tails, Gln^{24} of H2A and Gln^{19} of H3, have a positively charged residue in positions +5 and +4, respectively. Gln^6 of H2A, which is not appreciably modified, possesses two glycines next on the C-terminal side. On the other hand, Gln^{22} of H2B contains lysine residues in positions +1, +2, and +5. Positively charged residues in positions +1 and +2 do not occur in any case in the modified glutamines present in the N-terminal tails, and when the analysis includes all the modified glutamines, only Gln^{93} of H4, which is a poor substrate, possesses a single arginine in position +2. It is tempting to speculate that positive charges in positions +1 and +2, rather than in other positions, act as a discouraging feature for the TGase reaction, at least in unfolded protein regions. If this were the case, it would explain why Gln^{22} of H2B becomes a TGase substrate in nucleosomes. Actually, the charges of the lysines next to glutamine are surely neutralized by interaction with DNA, and in this way, an otherwise nonreactive glutamine residue becomes reactive.

Two other glutamine residues in the N-terminal tails, namely Gln^5 and Gln^{19} of H3, are modified in nucleosomes, but the rest of the glutamines modified in free histones are not labeled in nucleosomes at low ionic strength. Gln^{125} of H3 and Gln^{95} of H2B are in equivalent positions within helix III of the histone fold (30), and Gln^{93} of H4 is also placed in helix III. The remaining glutamines unlabeled in the nucleosome are either in the N- or C-terminal unstructured regions of the histones in the core particle (3). Anyway, our results show that the reactivity of some glutaminyl residues is lost in the nucleosome, due either to the proper folding of the histone molecules to form the octamer or to blocking by the proximity of other histones or DNA. Therefore, the TGase reaction is a useful probe to determine the accessibility of glutamine residues in the nucleosome. The possibility of using TGase to analyze protein structure and organization was foreseen by Folk (9), although he reported that the failure of many proteins to act as TGase substrates can lead to disappointing results. Fortunately enough, this is not the case with histones, and experiments to apply this methodology to the study of conformational changes in the nucleosome are being carried out in our laboratory.

The experiments described in this paper also show that DNC-modified histones can be properly reconstituted into core particles (see Fig. 6). The yield of reconstitution is obviously higher with histones modified in the N-terminal tails, which do not participate in histone-histone interactions, but the differences in the reconstitution yield do not mean that the reconstituted particles are defective. Therefore, nucleosome reconstitution with DNC-modified histones represents another potential use of the TGase reaction because the fluorescent probe may be a useful tool for structural studies.

It is somewhat surprising that histones are such good TGase substrates. First, it is widely accepted that only a minor fraction of proteins are glutaminyl substrates of TGase (10). Early data show, for instance, that only 3 out of 300 proteins observed in lymphocytes incorporate polyamines by the TGase-catalyzed reaction (31). Moreover, the number of reactive glutamines in a protein substrate is usually very limited. Takagi *et al.* (32) have recently found that out of ~40 glutamine residues in the propolypeptide of bovine von Willebrand factor, only four are substrates of factor XIIIa. Moreover, most of the proteins that act as TGase glutaminyl substrates *in vitro* are also physiological substrates. A few proteins and natural peptides, for which no physiological function for the TGase-catalyzed reaction has been described, are glutaminyl substrates *in vitro*, but it cannot be excluded that a function might be eventually found. On the other hand, the modification of Gln^{95} of H2B (Fig. 2*B*) is faster than that of many known natural substrates of the enzyme. For instance, the incorporation of putrescine into the propolypeptide of von Willebrand factor is completed only after 6 h of incubation with factor XIIIa (33), and the type 2 plasminogen activator inhibitor reacts even more slowly in the presence of tissue TGase (34). As other histone glutamines are modified even faster than Gln^{95} of H2B, it is tempting to wonder whether histones actually are natural substrates of TGase.

However speculative, there are several possibilities that the TGase-catalyzed histone modification plays a physiological role. For instance, TGases are involved in apoptosis, probably by cross-linking proteins to give apoptotic bodies (35). As chromatin is progressively condensed in perinuclear regions during apoptosis (see Ref. 36 for a review), it may be that TGase-catalyzed histone cross-linking mediates this chromatin condensation. An increase in TGase activity during apoptosis has been detected only in the cytoplasm (35), but it has been suggested that, due to cell shrinkage and cytoskeletal alterations, cytosolic enzymes may act on the nuclear components (36). Moreover, the possibility exists that TGase is present in some nuclei. For instance, it has been recently shown that apoptotic and non-apoptotic nuclei from rat intestinal mucosa cells immunoreact with a TGase antiserum (37). Preliminary experiments carried out in our laboratory also seem to indicate that chromatin condensation involving cross-linking of histones occurs upon incubating isolated chicken erythrocyte nuclei with TGase. It is worth noting that the existence of a cross-link between a glutamine of H2B and a lysine of H4 has been recently found in the sperm of the starfish *Asterina pectinifera* (38), thus providing support to our idea that the TGase reaction may occur in histones *in vivo*. Finally, putrescine is readily linked to histones, and the covalent attachment of polyamines to histones might also be of physiological significance. Some polyamine-binding proteins, presumably via the TGase-catalyzed reaction, have been described (39, 40), and polyamines have been suggested to modulate chromatin structure and function (41). It may be also possible that this modulation takes place after covalent binding, a mechanism suggested to occur in the modulation of ornithine decarboxylase (42). Obviously, more in-depth research on these topics will be necessary to investigate the above possibilities.

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