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Protein kinase in HeLa nucleosomes: a reevaluation of the interactions of histones with the ends of core particle DNA

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ABSTRACT

HeLa chromatin core particles contain a protein kinase which transfers phosphate from ATP to both nonhistone proteins and histones. The enzyme preferentially modifies H3 among the histones; about 7% of the H3 molecules in the nucleoprotein are modified at saturation. Activity of this kinase likely contributed to earlier results using crosslinking methodology to study which histones interact with the ends of core particle DNA. When the kinase is largely removed by sedimentation of core particles through sucrose gradients containing 0.45 M NaCl, crosslinking of the 5'-terminal label on DNA is observed only to histone H3. The overall efficiency of the crosslinking reaction is about 15%. The origin of the 5'-terminal ^{32}P previously assigned as crosslinked to H4 is not explained by the current experiments.

INTRODUCTION

As one approach to localizing histone-DNA interactions in chromatin core particles, I previously applied the crosslinking procedure of Levina and Mirzabekov [1] to HeLa nucleosomes bearing a 5'-terminal ^{32}P label [2]. Briefly, the procedure was as follows. Core particles were labeled at the 5'-DNA termini using polynucleotide kinase and γ-^{32}P-ATP. Guanylyl residues were methylated with dimethyl sulfate and the modified bases were depurinated at pH 6.0. The resulting aldehyde interacted with a nearby histone lysyl residue as a Schiff base; this species was reduced with borohydride to form a stable covalent DNA-protein crosslink. After digestion of the DNA with nucleases, the histones were separated by polyacrylamide gel electrophoresis and the histone neighboring the 5'-end of the DNA determined by autoradiography. The results demonstrated nearly equal amounts of ^{32}P apparently associated with histones H3 and H4.

In later experiments, I noticed variations in the proportions of label apparently associated with the two histones, usually in the direction of more label being present in H3. This finding prompted me to reassess the labeling and crosslinking procedure. The results presented here show that core par-

ticles from HeLa cells contain an endogenous protein kinase. Activity of this enzyme likely contributed to the results in the earlier crosslinking study. Removal of the bulk of the protein kinase activity by salt washing the core particles leads to detection of apparent crosslinking of the 5'-terminal ^{32}P label only to histone H3.

EXPERIMENTAL SECTION

Growth of HeLa cells and isolation of nuclei, chromatin and core particles were as previously described [3]. To remove the endogenous protein kinase, core particles were made 0.45 M in NaCl and sedimented in isokinetic sucrose gradients (C_m = 5%, isokinetic for particles with density 1.51 gm/cm^3 and at 4°C) containing 0.45 M NaCl, 1 mM EDTA, pH 7.0. Sedimentation was for 16 hr at 40,000 rpm in a Beckman SW41 rotor. The monomer peak was collected, dialyzed against 0.5 mM EDTA, pH 7.0, concentrated by dialysis against dry Sephadex, redialyzed and frozen until use.

5'-end labeling of core particles with γ-^{32}P-ATP (New England Nuclear Corp.) and polynucleotide kinase (Boehringer-Mannheim Corp.) was as previously described [4]. Assay of the endogenous protein kinase was carried out by incubation of core particles ($A_{260\ nm}$ = 10) with γ-^{32}P-ATP in 10 mM Tris-Cl, pH 7.5, 5 mM dithiothreitol, 5 mM $MgCl_2$ at 37°C for 20 min, except where indicated. Samples to be crosslinked were separated from unreacted ATP by gel filtration, using a 1.2 x 20 cm column containing Sephacryl S-200 (Pharmacia Corp.) in 0.05 M EDTA, 0.01 M sodium cacodylate, pH 7.0, at 20°C.

Methylation of nucleosomes was carried out for 16 hr at 4°C with stirring in the above buffer, using 5 µl dimethyl sulfate/ml sample. After modification, samples were dialyzed against 0.1 M NaCl, 0.05 M EDTA, 0.01 M sodium phosphate, pH 6.0, at 4°C. Depurination was performed at 50°C for 4 hr. After depurination, samples were cooled to 0°C and reduced for 30 min with 0.1 volume of freshly prepared 1.5 M sodium cyanoborohydride; the samples were then dialyzed to water and lyophilized. Various controls included samples not methylated, samples held at 0°C for the time required for depurination, samples not reduced, etc. as indicated in the text.

Two methods were used to remove the DNA which was not crosslinked to proteins. In one, lyophilized samples were dissolved in 20 mM Tris-Cl, pH 8.0, 20 mM $MgCl_2$, 5 mM $CaCl_2$, 1000 U/ml DNAase I (Worthington Corp.) and incubated at 37°C for 60 min. The samples were then lyophilized and utilized directly for histone separation. In the second method, carrier bovine serum albumin was added to samples before reduction. Dialyzed, lyophilized samples were

dissolved in water at about 1 mg protein/ml; 0.1 volume of 100% (w/v) trichloroacetic acid was added and samples were heated at 100°C for 15 min. After cooling to 0°C and incubation for over 2 hr, proteins were pelleted at 12,000 xg for 5 min, washed with a small volume of cold ethanol, repelleted and dried.

Samples were dissolved in sample buffer, heated at 100°C for 2-4 min and electrophoresed on discontinuous sodium dodecyl sulfate - 18% polyacrylamide gels as previously described [2]. Gels were stained, destained and subjected to autoradiography as previously described [2]. Gels and autoradiograms were scanned with an E-C Instruments Corp. densitometer. Integration of peak areas was performed by planimetry using a Numonics Corp. graphics calculator. To minimize any possible contributions of labeled proteins migrating near H3, integration of densitometric scans at the autoradiograms was done using a bandwidth equal to the bandwidth of H3 on a stained gel, subtracting baseline contributions to either side of the band. Autoradiograms were generally exposed and quantitated at two times to ensure linearity of the integrated peak areas with the amount of radioisotope.

RESULTS

Figure 1 shows the autoradiogram obtained after gel electrophoresis of reaction mixtures of core particles incubated with γ-^{32}P-ATP in the absence of (Figure 1A) or the presence of polynucleotide kinase (Figure 1B). In the absence of the exogenous enzyme, there is clearly incorporation of the isotope into histones, primarily H3, as well as into a number of nonhistone proteins present in the core particle preparation. When polynucleotide kinase is added, the bulk of the label is incorporated into core particle DNA; the amount of label associated with protein is somewhat reduced, presumably due to competition between the two kinases for the substrate ATP. In several preparations of core particles from HeLa cells, the total amount of ^{32}P incorporated in the absence of polynucleotide kinase was 15-20% of that incorporated when polynucleotide kinase was present. Thus, core particles from HeLa cells contain an active protein kinase which preferentially modifies H3 among the four smaller histones.

The autoradiogram shown in Figure 2 shows the results of several experiments which characterize features of the activity of this protein kinase. The time course of the modification is shown in slots j-o and is quantitated for H3 in Figure 3. Incorporation reaches saturating levels within about 30 min of incubation; the lack of further incorporation is not due to exhaustion of substrate ATP (data not shown). At the conclusion of the reaction, about 7%

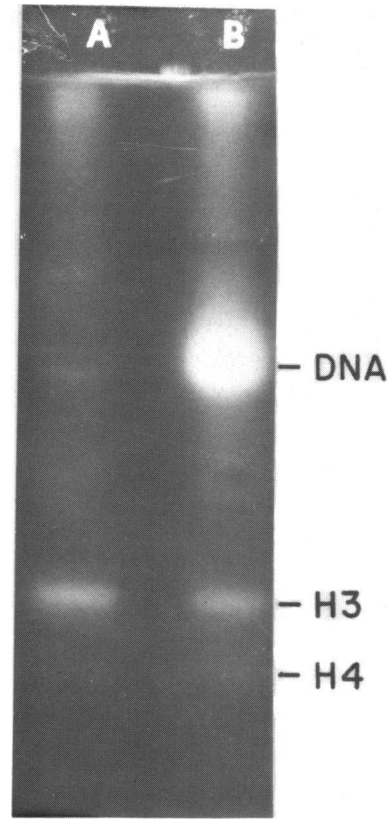

Figure 1. Autoradiogram of reaction products following incubation of HeLa core particles with γ-^{32}P-ATP in the absence (A) or presence (B) of polynucleotide kinase. After modification, reaction products were dissociated in 1% sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis and autoradiography of the gel. The positions of migration of 140 base length single stranded DNA and histones H3 and H4 are shown. Migration was from top to bottom.

of the histone H3 molecules present in the core particle preparation have been modified, assuming one phosphate incorporated per histone molecule.

The endogenous protein kinase requires magnesium ion for optimal activity. Addition of EDTA abolishes enzyme activity (Figure 2 a,b). Kinase activity is absent when calcium is substituted for magnesium (Figure 2 d) and is less than optimal in the presence of manganese (Figure 2 c) or cobalt (Figure 2 e). The isotopic substrate does not contain a radiochemical impurity which is transferred to histones; reduction of the specific radioactivity of the labeled ATP by addition of increased amounts of cold ATP leads to decreased incorporation of the label into proteins (Figure 2 a,f-h).

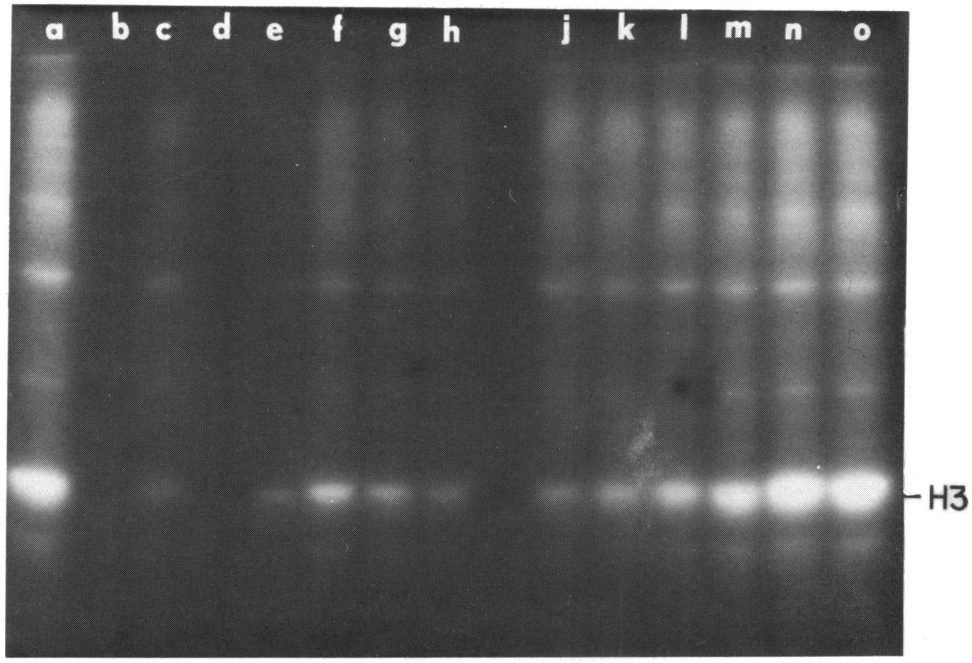

Figure 2. Characterization of the endogenous protein kinase in HeLa core particles. HeLa core particles were incubated with γ-^{32}P-ATP under various conditions. Reactions were terminated by addition of sodium dodecyl sulfate to 1% and products separated by gel electrophoresis and analyzed by autoradiography. The position of migration of H3 is noted. Migration was from top to bottom. (a) standard reaction, 20 min, (b) lacking magnesium, + 10 mM EDTA. (c-e) lacking magnesium, + 5 mM Mn^{+2} (c), Ca^{+2} (d) or Co^{+2} (e). (f-h) in addition to ^{32}P-ATP, unlabeled ATP was added at 3, 7, or 15 times the concentration of the isotopically labeled substrate, respectively. (j-o) time course of the reaction; incubation was terminated after 2.5, 5, 10, 20, 30, or 40 min. respectively. Equal amounts of histones were applied to all slots.

Sedimentation of HeLa core particles through sucrose gradients containing 0.45 M NaCl removes a major portion of this protein kinase. For such salt washed particles, less than 2% of the total ^{32}P incorporation observed in the presence of polynucleotide kinase is due to the endogenous protein kinase. Thus, over 90% of the protein kinase activity is removed by such sedimentation. To further reduce the contribution of the protein kinase to crosslinking experiments, these salt-washed particles were incubated with cold ATP (12 µM) prior to addition of ^{32}P-ATP (also 12 µM), either with or without polynucleotide kinase.

Using these salt-washed, preincubated core particles, I have repeated the crosslinking experiments previously described [2]. In all cases, the particles

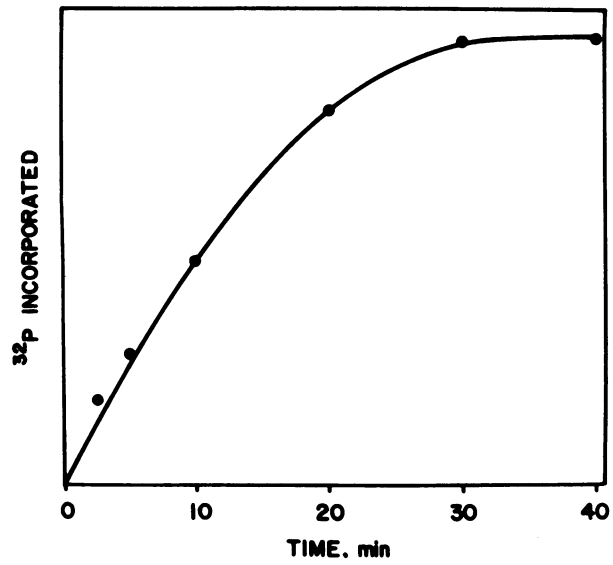

Figure 3. Time course of labeling of H3 by the endogenous protein kinase in HeLa core particles. Autoradiographs of the gel samples shown in Figure 2 (j-o) were scanned and areas migrating with H3 determined by planimetry. The time course of the incorporation of isotope into H3 is plotted, with an arbitrary scale for the ordinate. At the conclusion of the modification, scintillation counting of a sliced gel demonstrated that about 7% of the H3 population contained ^{32}P, assuming one phosphate per histone molecule.

incubated in the presence of ^{32}P-ATP, but in the absence of polynucleotide kinase, served as a primary control sample; any protein labeled in these samples derives from residual protein kinase activity and not from crosslinking to the DNA 5'-terminal label. Figure 4 shows the autoradiogram of a histone gel obtained in a typical experiment. The experiment depicted used hydrolysis of DNA in hot trichloroacetic acid; equivalent results were obtained with enzymatic degradation of the nucleic acid, although histone recoveries were better and background radioactivity reduced in the samples subjected to chemical hydrolysis of the DNA.

The two gel slots to the left represent labeling due to the endogenous protein kinase. Approximately equal amounts of ^{32}P are associated with both samples; (A) a control, methylated, but held at 0°C during the time required for depurination and (B) a sample depurinated at 50°C for 4 hr. The two samples to the right were labeled with γ-^{32}P-ATP and polynucleotide kinase. Both were methylated and then sample (C) was held at 0°C while (D) was depurinated at 50°C. When compared to the samples labeled only by the residual endogenous protein kinase, the samples treated with polynucleotide kinase have

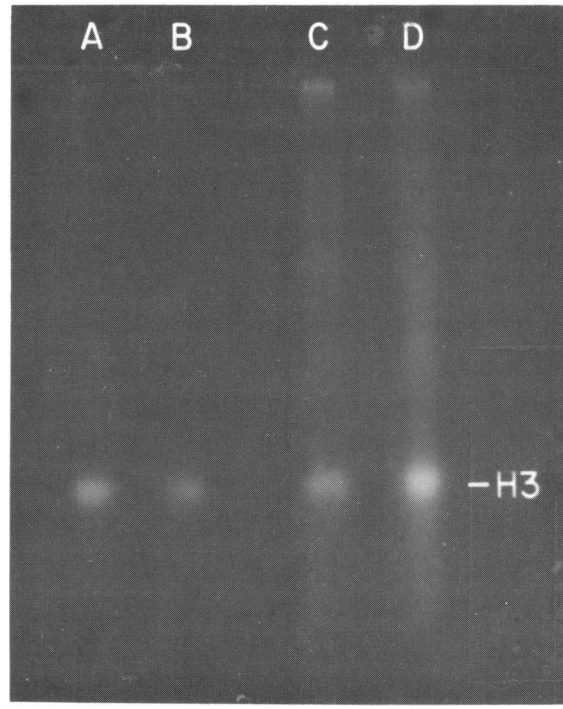

Figure 4. Autoradiogram of crosslinked 5'-terminal labeled core particles and controls. Salt washed HeLa core particles were preincubated for 15 min with cold ATP and then incubated for 15 min with ^{32}P-ATP alone (A,B) or with ^{32}P-ATP and 50 U/ml polynucleotide kinase (C,D). All samples were separated from unreacted ATP by gel filtration, methylated with dimethyl sulfate and dialyzed into the depurination buffer. Control samples (A,C) were held at 0°C while samples to be depurinated (B,D) were incubated for 4 hr at 50°C. All samples were reduced with cyanoborohydride, prepared for electrophoresis by hot trichloroacetic acid hydrolysis, subjected to gel electrophoresis and autoradiography. The position of migration of H3 is noted. Migration was from top to bottom. Approximately equal amounts of histones were applied to all slots.

a broadly distributed region of radioactivity migrating more slowly than the four smaller histones. The amount of material present in this region is not much altered by depurination conditions, hence it likely does not represent crosslinked DNA. Isotope in this region is not present in samples which are not methylated, but are subjected to the depurination incubation and reduction with cyanoborohydride. The label in this region is associated with protein; it is not removed by DNAse I or venom phosphodiesterase digestion or trichloroacetic acid hydrolysis, but it is removed by trypsin or proteinase K. The origin of the label in this area of the gel is not understood currently.

Radioactive phosphate is also associated with histone H3 in the samples subjected to the crosslinking procedure. The amount of ^{32}P migrating with H3 in the control (not depurinated) sample (Figure 4C) appears similar visually to that associated with H3 in the other control samples, modified in the absence of polynucleotide kinase (Figure 4A,B). In contrast, more label migrates with H3 in the sample both modified with polynucleotide kinase and depurinated and crosslinked (Figure 4D). Densitometric scans of these samples confirm this visual impression; the data are detailed in Table I.

Compared to the amount of isotope associated with H3 in the first control sample, modified in the absence of polynucleotide kinase and maintained at 0°C, 80% of the radioactivity is retained in the second control sample, similarly modified but subjected to the depurination conditions of 50°C, pH 6.0, 4 hr. A third control sample, modified with polynucleotide kinase but not depurinated, has a slightly lower amount of ^{32}P comigrating with H3 than its partner, the first control sample, consistent with the autoradiogram shown in Figure 1. The fourth sample, modified with polynucleotide kinase and depurinated, has nearly five times the amount of isotope associated with H3 as does the second control sample, also depurinated but modified only by the residual protein kinase. Thus, the amount of ^{32}P comigrating with H3 is nearly identical for the three control samples, and is increased several-fold for the experimental sample, where crosslinking of the terminal ^{32}P-DNA label to protein would be expected to have occurred.

The efficiency of the crosslinking reaction was calculated as follows. Incorporation of ^{32}P in the absence of polynucleotide kinase was about 1% of

TABLE I

Amount of ^{32}P Associated With Histone H3

Labeling by	0°	50°
Residual protein kinase	1.0	0.8
Residual protein kinase + polynucleotide kinase	0.9	3.8

Densitometric scans of the autoradiograms shown in Figure 4 and stained gels were quantitated by planimetry using a graphics calculator. Arbitrary ratios of the intensities of the autoradiographic peaks to the stained bands are presented here. The two columns are temperatures used during the depurination incubation. The quantity of ^{32}P comigrating with H3 is expressed relative to a value of 1.0 for the sample modified only by the residual protein kinase and held at 0°C during the 4 hr required for depurination.

that observed in the presence of the exogenous enzyme. Since the amount of ^{32}P migrating with H3 in the experimental sample (Figure 4D) is at least four times that in the appropriate control sample (Figure 4B), 4% - 1% = 3% of the 5'-terminal label has apparently been crosslinked to histone H3. Since, statistically, 20% of the 5'-terminal residues in the core particle are guanylyl residues, the efficiency of the crosslinking is 3%/20% = 0.15.

DISCUSSION

The protein kinase identified in these preparations of HeLa core particles bears similarities to that previously described by Shoemaker and Chalkley in chromatin; preferential modification of H3, among the histones, and dissociability at salt concentrations in excess of 0.2 M NaCl [5]. The presence of the kinase in isolated core particles was unexpected and suggests caution in studies of chromosomal subunits which involve enzymatic modification or degradation. While we and others have noted the presence of non-histone proteins in core particle preparations [3,6], the possible effects of these proteins on structural features of the isolated nucleosomes have largely gone unnoticed. Even more problematic are instances, such as the current one, where an enzyme, present in catalytic quantities, can have major effects on the outcome of labeling experiments. Here, the presence of the protein kinase in core particles has complicated attempts to assign the histones which neighbor the ends of core particle DNA.

In the current experiments, I have used salt washing and preincubation with cold ATP to reduce the incorporation of ^{32}P due to the residual protein kinase to less than 1% of the total isotope incorporation in the presence of polynucleotide kinase. All controls contain approximately similar amounts of ^{32}P migrating with histone H3; the experimental sample, labeled with polynucleotide kinase and depurinated after methylation, has about four times as much ^{32}P associated with H3 as any of the controls. No modification of the other three smaller histones has been noted in these salt washed core particles.

Thus, it appears that H3 can be crosslinked to the 5'-terminal ^{32}P-DNA label in modified core particles. While the increment in the amount of label associated with H3 is somewhat small compared to the residual labeling due to the protein kinase, the estimate of the amount of label has been made conservatively and the efficiency of the crosslinking reaction is not unreasonable in view of the several steps of the reaction and the necessity for close proximity of the two reacting groups in order for crosslinking to occur. Alternatively, it is possible that other histones interact with the ends of

DNA in most core particles, but that the region of the histone(s) near the 5'-end of the nucleic acid does not contain an amino group close enough to the end of the DNA to allow crosslinking to occur. It seems likely that resolution of this question, together with numerous others about the precise arrangements of proteins and DNA in the chromatin subunit, will likely have to await higher resolution x-ray diffraction studies of core particle crystals containing intact histones.

Assuming that the activity of the protein kinase in the three preparations of core particles used for my previous study [2] was similar to that observed for the several preparations used in the current investigation, it is apparent that nearly all the "crosslinking" detected previously must have derived from the activity of this enzyme and not from actual crosslinking of the DNA label to protein. Thus, about 20% of the total ^{32}P incorporated into core particles not subjected to salt washing is due to the activity of the protein kinase; crosslinking of about 3% of the total incorporated ^{32}P would be difficult if not impossible to detect on top of the background protein label. In this regard, the virtual absence of label associated with H4 in the current studies contrasts sharply with the apparent association of about 50% of the label with this histone in the previous study [2]. It is possible that 1) very efficient crosslinking of the 5'-terminal DNA label to H4 could have occurred previously, but not be repeatable, 2) that those particles utilized previously might have had a different kinase or an H4 more susceptible for phosphorylation, or 3) that proteolytic degradation of H3 labeled by the protein kinase may have occurred in the previous experiments. None of these alternatives seem very likely; the origin of the ^{32}P label apparently associated with H4 in the previous experiments remains an enigma.

If indeed H3 does interact with the ends of DNA in isolated core particles, a conclusion suggested but not proven by these studies, interpretation of the crosslinking results in terms of core particle structure is simplified compared to the previous suggestion that both H3 and H4 bound to the ends of DNA in nucleosomes. Association of only one histone with the ends of DNA in the core particle is consistent with a symmetrical array of proteins in the core of the nucleosome; an array thought likely on the basis of theoretical considerations [7] and supported by elements of symmetry in the path of DNA around the protein nucleus of the core particle [8]. Additionally, as previously noted [9], association of H3 with the ends of core particle DNA is quite consistent with the central role played by this histone, in concert with H4, in organization of the basic folding of DNA in the chromatin subunit particle [9-11].

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