Hyperacetylated histones facilitate chromatin assembly *in vitro*

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ABSTRACT

We have examined the effect of histone acetylation on the *in vitro* assembly of nucleosomes with DNA and purified histones at physiological ionic strength in the presence of polyglutamic acid. We have found that hyperacetylated histones assemble nucleosomes with greater efficiency, and to a greater extent, than either control or hypoacetylated histones. Assembly reactions were performed over a range of histone to DNA ratios (0.25 to 3.0, w/w) and polyglutamic acid to histone ratios (0 to 1.6, w/w). Although polyglutamic acid may act as a sink to prevent nonspecific histone-DNA interactions, our data suggest that the polyanion primarily facilitates the assembly of nucleosomes by organizing histones into a form that is amenable to deposition.

INTRODUCTION

The arrangement and organization of chromosomal proteins may play an important role in modulating gene expression. Consequently an understanding of the process by which chromatin is assembled may well reveal important features of this control. Since it has proved to be very difficult to follow this process in the cell, a number of attempts have been made to assemble chromatin-like structures *in vitro* (reviewed in 1). However, since there are strong ionic interactions between histones and DNA, it is difficult to introduce both species alone into a reaction vessel without the occurrence of precipitation, which severely inhibits appropriate assembly.

Initial attempts at *in vitro* assembly of chromatin involved the use of urea and/or high concentrations of sodium chloride in order to limit the precipitation reaction. While these methods were somewhat successful in reconstituting nucleosome-like structures on linear DNA they do not generate regular spacing and they can obviously reflect very little of the assembly processes that occur *in vivo*. Several methods have since been developed that allow the formation of nucleosome-like structures at physiological ionic strengths (100-200 mM NaCl). Polyanionic compounds such as polyglutamic acid and polyaspartic acid can serve to facilitate nucleosome

formation at physiological ionic strengths (2,3). An acidic protein has been isolated from *Xenopus* oocytes that can be shown to facilitate nucleosome formation (4,5,6,7). Furthermore, it has also been argued that nucleosome formation can proceed at physiological ionic strengths, in the absence of facilitating factors (8,9,10,11).

We have analysed those factors necessary for optimum nucleosome reconstitution, particularly concentrating on reassembly facilitated by polyglutamic acid. We have found that three factors are important for optimum nucleosome formation: the acetylation state of the histones, the histone to DNA ratio, and the polyglutamic acid to histone ratio. By systematically altering these three factors we have determined reconstitution optima for hyperacetylated, control and hypoacetylated histones. The hyperacetylated histones reconstitute at a higher efficiency than the other two histone types under most conditions. This observation is of interest because it has been shown that newly synthesized H4 is almost exclusively diacetylated and that a significant proportion of newly synthesized H3 is acetylated at the time of deposition (12,13,14).

MATERIALS AND METHODS

Preparation of Chromatin.

Mid-log phase HTC cells were treated with the appropriate sodium butyrate regime to induce either histone hyperacetylation (6 mM butyrate for 6 hours followed by 50 mM butyrate for 12-14 hours) or histone hypoacetylation (10 mM butyrate for 24 hours followed by 6 hours in fresh medium without butyrate) essentially as described previously (15). The cells were harvested and stored at -70°C until use. Frozen cells were thawed, disrupted in a dounce homogenizer in wash medium (0.25 M sucrose, 1% Triton, 0.01 M magnesium chloride, 0.005 M sodium butyrate, 0.01 Tris pH 7.4) and the nuclei were collected by centrifugation. Nuclei were washed twice in wash medium and twice in 0.010 M EDTA, 0.005 M sodium butyrate, 0.010 M Tris-HCl, pH 7.4 to release the chromatin. The final chromatin pellet was suspended in water and dispersed by sonication.

Hydroxyapatite Chromatography.

Core histones, free of histone H1, were prepared from hyperacetylated, control or hypoacetylated chromatin using hydroxyapatite chromatography (16,17). An equal volume of 0.3 M NaCl, 0.01 M mercaptoethanol, 0.005 M sodium butyrate, 0.08 M sodium phosphate, pH 6.8 was added to the chromatin sample and the material was applied to a hydroxyapatite column (5 mL bed

Figure 1. The acetylation levels of core histones used for reconstitution of chromatin. Histone samples were precipitated with an equal volume of saturated Reinecke salt in 0.5 M HCL, washed with acetone/conc. HCL, (98:2) and resolved by electrophoresis on a 12.5% acrylamide gel containing 8M urea and 0.44% Triton X-100. Lane 1, H1 from control cells; lane 2, hypoacetylated core histones; lane 3, control core histones, lane 4, hyperacetylated core histones.

volume/mg chromatin) equilibrated with the same buffer. The column was washed with 1.5 column volumes of the equilibration buffer, histone H1 was then eluted with 1.5 volumes of 0.6 M NaCl, 0.01 M mercaptoethanol, 0.005 M sodium butyrate, 0.08 M sodium phosphate, pH 6.8 and the core histones were eluted with 1.5 volumes of 2.0 M NaCl, 0.01 M mercaptoethanol 0.005 M sodium butyrate, 0.08 M sodium phosphate, pH 6.8. The elution pattern was determined by measuring the absorbance of column fractions at 230 nm. The appropriate fractions were pooled, dialyzed against 0.15 M NaCl, 0.01 M mercaptoethanol, 0.005 M sodium butyrate and 10 mM Tris-HCl, pH 7.6, and stored at -70°C .

Gel Electrophoresis.

The purity and acetylation state of core histone samples were assayed with Triton/acid/urea electrophoresis gels containing 8 M urea and 0.44% Triton X-100 as described by Alfageme *et al.* (18) (see figure 1). Gels were stained in methanol/acetic acid/water (4:1:5) containing 0.25% coomassie blue.

Quantitation of Protein.

The protein concentrations of histone samples were determined by the method of Lowry (19) using as a standard, a solution of calf thymus histones that had been prepared from acid dried material. Relative protein concentrations were ascertained by comparing densitometric scans of coomassie blue-

Figure 2. The resolution capabilities of 1% vs 2% agarose electrophoresis. Samples were reconstituted as described in the Methods section. Lanes 1, 2 and 3 correspond to material reconstituted to different extents. Lanes marked r contain the topoisomerase treated pBR322 DNA used for reconstitution; lanes marked m contain pBR322 prior to topoisomerase treatment. Electrophoresis was performed in either 2% agarose (A) or 1% agarose (B) TPE gel as described in the text.

stained 15% acrylamide electrophoresis gels containing 0.1% SDS.

Reconstitution of Chromatin.

Form II pBR322 was prepared by incubating the DNA with topoisomerase I (BRL, 2 units of topoisomerase/ μ g DNA) at 37°C for 40 minutes at 0.05 mg/ml DNA in 150 mM NaCl, 10 mM Tris, pH 8.0. This relaxed DNA, still containing the topoisomerase activity, was added to a histone/polyglutamic acid mixture, also in 150 mM NaCl, 10 mM Tris, pH 8.0, to a final DNA concentration of 10.0 μ g/ml. The sample was incubated for 60 minutes at 37°C, centrifuged for 5 minutes at 15,000 X g and the supernatant and pellet were separated for subsequent analysis. Poly-L-glutamic acid (average MW 50,000) was obtained from Sigma; concentrations of polyglutamic acid solutions were determined from their absorbance at 230 nm based on an absorbance of 0.9 for a 1 mg/ml solution.

Figure 3. Centrifugation removes incompletely packaged material. The three sets of lanes marked A, B and C contain the DNA from reconstitutions performed to different extents. Lanes marked t contain the total DNA distribution present prior to centrifugation, lanes marked p and s contain the DNA distribution present in either the pellet or the supernatant after a 5 minute, 15,000 X g centrifugation of the reconstituted material. Lanes r and m contain the relaxed and marker pBR322 samples as in figure 1.

Supercoiling Assay.

Nucleosome reconstitution reactions were assayed by following the induction of supercoils that occurs when a nucleosome is formed on a closed circular DNA molecule. The reconstitution reaction was terminated by adding sodium acetate, pH 8.0 to a final concentration of 0.3 M. Proteinase K and SDS were then added, to final concentrations of 0.2 mg/ml and 0.5% and the sample was incubated at 37°C for 40 minutes. The DNA in the sample was then precipitated with ethanol, collected and analyzed on a 2% agarose gel in 0.030 M sodium phosphate, 0.036 M Tris, 0.001 M EDTA, pH 7.7 (TPE) at 2 V/cm for 24-26 hours (20). The gel was stained with ethidium bromide and the DNA was visualized with short-wave UV light. The DNA pattern was photographed with Polaroid type 55 film and the distribution was analysed by scanning the negative with a Beckman DU-8 spectrophotometer.

RESULTS

A Sensitive Assay of DNA Supercoiling.

Most published reconstitution systems use a 1% agarose electrophoresis system to assay the number of supercoiled species induced by nucleosome for-

mation. However, a 1% agarose gel is capable of resolving only up to 14 different supercoiled species of pBR322, whereas a 2% agarose gel is capable of resolving up to 22 different supercoiled species. Thus the resolution pattern of a 1% agarose gel can be misleading. In figure 2B, analysis on a 1% agarose gel suggests that at their maximum extent, samples 1, 2 and 3 appear to be as fully supercoiled as pBR322 extracted from bacterial cells (sample m). When these same samples were resolved on a 2% agarose gel (figure 2A) it is apparent that the distribution of supercoiled species in sample 1 approaches that of pBR322, that the distribution of supercoiled species in sample 3 is centered about a species with 2-3 fewer supercoils than that of sample 1, and that the distribution of supercoiled species in sample 2 is centered about a species with even fewer supercoils. The results of a 1% agarose gel analysis could lead one to conclude that an in vitro reconstitution system is producing a fully packaged DNA molecule when in fact, the DNA is incompletely packaged.

Aggregation Limits Reconstitution.

A major limitation to appropriate nucleosome assembly is the formation of aggregated material. We have included a centrifugation step at the end of the reconstitution period in order to remove aggregated material resulting from inappropriate histone-DNA interactions. Material was reconstituted under conditions that produce a large proportion of highly packaged material, as assayed by supercoil induction (figure 3A), under conditions that produce slightly less packaging (figure 3B) and under conditions that produce a large proportion of unpackaged material (figure 3C). In figure 3, the DNA distribution present in the reconstitution before the centrifugation step is shown in lane t, and the DNA distributions present in the pellet and supernatant after the centrifugation are shown in lanes p and s. It is apparent that in each case, the sample before centrifugation contains a broad distribution of DNA species, the supernatant of each preparation contains the majority of the more fully supercoiled species (indicating a higher degree of nucleosome formation on that material) while the pellet of each preparation contains the bulk of the least supercoiled species, including a substantial amount of the completely unpackaged molecules present in the t sample. This suggests that the event that produces the aggregation of DNA-containing material is acting at the expense of nucleosome formation.

Reconstitution Using Variably Acetylated Histones.

We have studied the effect of various levels of acetylation on the ability of histone to facilitate reassembly on a circular DNA molecule,

assaying extent of appropriate assembly by following the insertion of supercoils. In the absence of any additional agents we find that assembly by histone alone is very inefficient with a maximum of only 7-8 supercoils inserted (figure 4A). Under these conditions we find that above a histone/DNA ratio of 1.25 essentially all the material is precipitated, with very little material (less than 10%) left in the supernatant fraction and with that material showing essentially no packaging. Even when assembly is performed at a histone/DNA ratio of 1.0, when approximately 30% of the hyperacetylated material is precipitated, the insertion of supercoils in the remaining soluble material is small. Thus, under the conditions of physiological ionic strength that we employ, the organization of the histone upon DNA following simple mixing of the two components alone, is at best imperfect. Factors in addition to the aggregation described in the previous section are functioning to limit assembly, and there is a need for some form of histone organizing system.

In the presence of polyglutamic acid as a histone organizer, the assembly process is much improved (3). At low polyglutamic acid/histone ratios we find a dramatic difference in behavior between hyperacetylated histone and control or hypoacetylated material. This is shown in figure 4B in which we see that in the presence of 0.44 mg of polyglutamic acid per mg of histone, hyperacetylated histone can insert up to 24 supercoils whereas control or hypoacetylated histone can only insert a maximum of 11 supercoils. Increasing the polyglutamic acid to histone ratio leads to an increased capacity of both control and hypoacetylated histones to package nucleosomes, although the packaging ability of control histones never attains that of hyperacetylated or hypoacetylated histones. We see that at polyglutamic acid to histone ratios of 0.89 and above (figure 4C, 4D and 4E) the hyperacetylated histone inserts a maximum of 20-23, with these maxima attained at histone to DNA ratios of 1.75 to 2.0. Hypoacetylated histones attain similar packaging maxima at polyglutamic acid to histone ratios of 0.89 and above, but these maxima are never attained below a histone to DNA ratio of 2.25. Control histones show a decreased packaging efficiency in two different ways: the maximum number of supercoils induced is never greater than 17 (attained at polyglutamic acid to histone ratios of 0.89 and 1.1) and these maxima are attained over a limited range of histone to DNA ratios. As the polyglutamic acid to histone ratio is increased above 1.1 we see a decrease in control histone packaging not observed with either hyperacetylated or hypoacetylated histones. With all three histone classes, we find that there

is a decrease in nucleosome formation at high histone to DNA ratio, and this decrease can be correlated with an increase proportion of material found in the pellet sample (data not shown), reflecting aggregation phenomena at high histone/DNA levels.

The data contained in figure 4 have been plotted in a different manner to reveal an important property of polyglutamic acid in this reconstitution system. The effect of varying the polyglutamic acid/histone ratio over a fairly wide range is shown for each class of histone in figure 5. Once again, we see that there is very poor packing in the absence of polyglutamic acid, and in the presence of high histone to DNA ratios for all three histone classes. An important trend becomes apparent when the data are plotted in this manner. We find that with hyperacetylated histones, a similar reconstitution maximum is obtained at the four polyglutamic acid to histone ratios, but this maximum is obtained at lower histone to DNA ratios as the amount of polyglutamic acid in the reaction is increased. We find that at a polyglutamic acid to histone ratio of 0.44, the maximum amount of reconstitution occurs at a histone to DNA ratio of 2.5, while at a polyglutamic acid to histone ratio of 1.6, the maximum is obtained at a histone to DNA ratio of 1.75. Somewhat similar but more muted effects are noted for control and hypoacetylated histones.

DISCUSSION

It is quite apparent that hyperacetylated histones are capable of assembling a greater number of nucleosomes than either control or hypoacetylated histones under most conditions. Furthermore, the assembly assay is highly reproducible. For example, DNA reconstituted with hyperacetylated histones at a histone:DNA ratio of 1.75, in the presence of 1.1 polyglutamic acid consistently induces the formation of 20 supercoils (mean = 20.3 ± 0.8 , $n = 6$). What is not apparent from the above data is the greater solubility of material reconstituted with hyperacetylated histones. If one examines

Figure 4. Nucleosome reconstitution varying the histone acetylation state at various histone to DNA ratios, using ratios of 0 (A), 0.44 (B), 0.89 (C), 1.1 (D) and 1.6 (E) polyglutamic acid to histone. The number of supercoils induced in the supernatant fraction from reconstitutions performed at five different polyglutamic acid ratios is plotted as a function of the histone to DNA ratio. The number of supercoils induced is the number of supercoils present in the DNA species determined to be at the center of the supercoil distribution. Supercoil distributions were obtained from densitometric scans of Polaroid type 55 negatives of the ethidium bromide stained, 2% agarose TPE gels.

Figure 5. Nucleosome reconstitution varying the polyglutamic acid to histone ratio, at various histone to DNA ratios with either hyperacetylated, control or hypoacetylated histones. The number of supercoils induced in the supernatant fraction of each reaction was determined as in figure 4.

the distribution of DNA between pellet and supernatant under various reconstitution conditions, one finds that material reconstituted with hyperacetylated histones always exhibits a greater solubility. For example, at a histone to DNA ratio of 2.25, in the presence of 1.56 units of polyglutamic acid per unit of histone, 70% of the DNA is present in the supernatant after reconstitution with hyperacetylated histones while the control and hypoacetylated histone produce 30% soluble material. In terms of *in vivo* requirements

Figure 6. The number of supercoils induced by nucleosome formation at constant histone to DNA ratios as a function of the polyglutamic acid to DNA ratio. The number of supercoils induced in the supernatant fraction of each reaction was determined as in figure 4.

for chromatin assembly, this increase in solubility associated with material reconstituted with hyperacetylated histones may be important.

We have considered the possibility that equal numbers of octamers of hyperacetylated and control histones bind to DNA but that the different histone classes induce a different linking number change as they form a nucleosome. This might reflect, for instance, a putative ability of the acetylated histones to wrap 145 bp of DNA more tightly about each octamer than either control or hypoacetylated histones, even though one might intuitively expect the reverse. In practice such a difference in behavior would be very difficult to measure. The two techniques which might elucidate this, electron microscopy or oligomer repeat length determination, are not likely to be sufficiently precise in the presence of even modest amounts of inappropriate assembly, since we do not know what the effects of this are upon appearance in the electron microscope or upon nuclease digestion. Although this kind of effect could account for the small differences in assembly between hyperacetylated and hypoacetylated histones at higher polyglutamic acid concentrations it seems unlikely that it can account for the 100% difference in assembly at lower polyglutamic acid concentrations. Accordingly we feel confident that the difference in behavior of the various histone types reported in this paper are a reflection of real differences in

packing ability, most likely due to variations in the relative amounts of appropriate and inappropriate packing.

It is possible that polyglutamic acid is merely functioning as a histone sink, slowly releasing histone to interact productively with DNA, while preventing the massive precipitation that would occur if the same quantity of histone were allowed to react with DNA in the absence of the polyanion. However, if polyglutamic acid were merely absorbing histone, one would expect to observe decreasing assembly at a given histone to DNA ratio as the polyglutamic acid to histone ratio is increased. As indicated in figure 6, in which the reconstitution performance is plotted as a function of increasing polyglutamic acid to DNA ratios at constant histone to DNA ratios, we see the opposite trend. We find that for all three histone classes, assembly increases as the amount of polyglutamic acid in the reaction increases, until very high concentrations of polyglutamic acid are used, at which point the polyanion appears to interfere with the assembly reaction.

There are several explanations for the superior assembly activity of the hyperacetylated histones. One possibility is that the chromatin product produced by the hyperacetylated histones has a greater solubility than either the control or hypoacetylated histone reconstitute. If this were the only factor then one might have expected to find that control histones assemble at a level intermediate to hyperacetylated and hypoacetylated histones. A possibility that reflects the intermediate assembly capacity of hypoacetylated histone is based on the observation that *in vivo*, newly deposited H3 and H4 are in a diacetylated form (a requirement for deposition which is partially fulfilled by our hyperacetylated histone preparation) but newly deposited H2A and H2B are found in a largely unacetylated form (a requirement which is met by the hypoacetylated histone preparation). The fact that control histones meet neither of these assembly requirements is consistent with their poorer assembly activity relative to hyperacetylated or hypoacetylated histones.

It has been reported that polyglutamic acid has the ability to organize core histones into octamers at physiological ionic strengths (3). It is not known, however, if the octamers formed by polyglutamic acid are released as free octamers before they interact with DNA or if the polyglutamic acid/histone octamer/DNA form a ternary complex during the nucleosome formation reaction. If the former case is correct, then the increased assembly activity of hyperacetylated histones may reflect an increased stability of

free octamer. If the latter reaction is the major route, the acetylation state of the histones may reflect the ability of a histone octamer to transfer from one polyanion to the other.

The use of polyglutamic acid as a chromatin assembly factor has the advantage that it provides a defined assembly system as opposed to using assembly extracts isolated from cells. On the other hand we eventually want to utilize those agents thought to modulate assembly in vivo. In this regard, nucleoplasmin has been proposed as an in vivo assembly protein, and we have found that hyperacetylated histones are also superior to control and hypoacetylated histones as assembly substrates for this agent, when it is prepared as described in the literature (5). However, the published procedure yields a nucleoplasmin preparation containing various amounts of an impurity which interferes with the assembly reaction. We are currently attempting to further purify nucleoplasmin, and until this is accomplished, polyglutamic acid appears to be a valuable model for nucleosome assembly.

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REFERENCES

1. Laskey, R.A. and Earnshaw, W.C. (1980) *Nature* 186, 763-767.
2. Nelson, S., Wiegand, R. and Brutlag, D. (1981) *Biochem.* 20, 2594-2601.
3. Stein, A., Whitlock, J.P. and Bina, M. (1979) *Proc. Natl. Acad. Sci. USA* 76, 5000-5004.
4. Laskey, R.A., Mills, A.D. and Morris, R. (1977) *Cell* 10, 237-243.
5. Laskey, R.A., Honda, B.M., Mills, A.D. and Finch, J.T. (1978) *Nature* 275, 416-420.
6. Earnshaw, W.C., Honda, B.M. and Laskey, R.A. (1980) *Cell* 21, 373-383.
7. Mills, A.D., Laskey, R.A., Black, P. and DeRobertis, E.M. (1980) *J. Mol. Biol.* 139, 561-568.
8. Ruiz-Carrillo, A., Jorcano, J.L., Eder, G. and Lurz, R. (1979) *Proc. Natl. Acad. Sci. USA* 76, 3284-3288.
9. Ellison, M.J. and Pulleyblank, D.E. (1983) *J. Biol. Chem.* 258, 13307-13313.
10. Ellison, M.J. and Pulleyblank, D.E. (1983) *J. Biol. Chem.* 258, 13314-13320.
11. Ellison, M.J. and Pulleyblank, D.E. (1983) *J. Biol. Chem.* 258, 13321-13327.

12. Ruiz-Carrillo, A., Wangh, L.J. and Allfrey, V.G. (1975) *Science* 190, 117-128.
13. Jackson, V., Shires, A., Tanphaichitr, N. and Chalkely, R. (1976) *J. Mol. Biol.* 104, 471-483.
14. Sealy, L. and Chalkley, R. (1979) *Arch. Biochem. Biophys.* 197, 78-82.
15. Covault, J., Sealy, L., Schnell, R., Shires, A. and Chalkley, R. (1982) *J. Biol. Chem.* 257, 5809-5815.
16. Bloom, K.S. and Anderson, J.N. (1978) *J. Biol. Chem.* 153, 4446-4450.
17. Simon, R.H. and Felsenfeld, G. (1979) *Nuc. Acids. Res.* 6, 689-696.
18. Alfageme, C., Zweidler, A., Mahowald, A. and Cohen, L. (1974) *J. Biol. Chem.* 149, 3729-3733.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
20. Shure, M. and Vinograd, J. (1976) *Cell* 8, 215-226.