Partial Purification, from *Xenopus laevis* Oocytes, of an ATP-dependent Activity Required for Nucleosome Spacing in Vitro*

(Received for publication, January 6, 1992)

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A critical feature of chromatin with regard to structure and function is the regular spacing of nucleosomes. In *vivo*, spacing of nucleosomes occurs in at least two steps, but the mechanism is not understood. In this report, we have mimicked the two-step process in *vitro*. A novel spacing activity has been partially purified from *Xenopus laevis* oocytes. When this activity is added, either at the beginning or at the end of a nucleosomal assembly reaction, it can convert a DNA template consisting of irregularly spaced nucleosomes into a chromatin structure made up of regularly spaced nucleosomes with a repeat length of about 165 base pairs. The reaction requires ATP. Histone H1 is able to increase the nucleosomal repeat from 165 to 190 base pairs. This two-step increase in nucleosomal repeat length suggests that both the spacing activity and histone H1 contribute to generating repeat lengths of greater than 165 base pairs and that their contributions may be additive. Alternatively, the critical step in the spacing reaction may not be the formation of the 165-base pair repeat but may be the sliding of nucleosomes or the reorganization of the octamer structure induced by the spacing activity.

In the nucleus of all eukaryotic cells, DNA is complexed with about an equivalent mass of histones and nonhistone proteins to form chromatin, which is a periodic structure made up of repeating, regularly spaced subunits, the nucleosomes (1). Recent genetic and biochemical evidence has demonstrated that a central role of chromatin is to maintain genes in a repressed state (reviewed in Refs. 2–4). In most cases, the formation of nucleosomes on the promoter of an eukaryotic gene prevents the establishment of a transcription complex. Therefore, mechanisms must exist enabling transcription factors to gain access to a promoter covered with nucleosomes. One postulated mechanism is that transcription factors can associate with DNA during replication when nucleosomes are disrupted (3). Other mechanisms involving direct nucleosome displacement and specific positioning of nucleosomes have also been suggested (4–7). Elucidation of these mechanisms, at the molecular level, is crucial to our understanding of gene expression and regulation during cellular differentiation.

In order to understand how a eukaryotic promoter is activated or repressed within a chromatin environment, it is first necessary to understand how DNA is assembled into chromatin. Because it is not possible to isolate sufficient amounts of newly replicated DNA for biochemical analysis, *in vivo* analysis of chromatin assembly is very difficult. Therefore, several *in vitro* chromatin assembly systems have been developed. Under very stringent experimental conditions, often employing nonphysiological conditions, purified histones and DNA can be combined to form nucleosomes (8–10). Although these purified systems have provided important information concerning nucleosome structure, they fail to reproduce one important feature of chromatin, the regular spacing of nucleosomes. In some instances, nucleosome spacing is observed when artificial DNA templates are used (11, 12). Cellular extracts prepared from *Xenopus laevis* oocytes or eggs (13–18) and HeLa cells (19–21) can assemble authentic chromatin *in vitro*, either from a replicating or nonreplicating template. These systems have recently demonstrated that the nucleosome assembly process occurs in two steps (17, 18, 22–28). Histones H3 and H4 first complex with DNA to form a subnucleosomal particle, and then H2A and H2B bind to form a nucleosome. A similar mechanism operates *in vivo* (29). To generate chromatin with physiologically spaced nucleosomes, ATP is required (13–15, 18, 21, 30).

*In vivo*, the spacing reaction occurs in at least two steps (31 and references therein). In the first step, nascent chromatin, which displays a shortened nucleosomal repeat length of approximately 165 bp and contains at least the histone octamer of H3, H4, H2A, and H2B, is assembled. In the second step, the longer spacing of mature chromatin is established. At present, nothing is known about this process or the process whereby the repeat length of mature chromatin varies between different cell types. Fractionation of the cellular chromatin assembly systems should reveal the mechanism of nucleosome spacing. The *Xenopus* oocyte system has a major advantage in fractionation experiments because chromatin assembly occurs very efficiently in the absence of DNA replication, reflecting the high concentration of chromatin assembly components in the oocyte. In contrast with the HeLa system, this allows separation of the two complicated processes, replication and chromatin assembly. The high concentration of assembly components in oocyte extracts also allows direct visualization of the reaction products without the need to label chromatin precursors. Previous attempts, however, to reconstitute nucleosome spacing *in vitro* from fractionated

*This work was supported in part by Grant 890420 from the National Health and Medical Research Council, Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetracetic acid; AMP-PNP, adeny1-5'-yl imidodiphosphate; HMG, high mobility group proteins; ATPyS, adenosine 5'-O-(thiotriphosphate); kb, kilobase(s).
Xenopus oocyte or egg extracts have been unsuccessful (23, 32, 33).

We have now been successful in restoring nucleosome spacing in vitro, with repeat lengths of up to 190 bp, using various chromatographic fractions isolated from X. laevis oocytes plus histone H1. One of these fractions contains a novel nucleosome spacing activity. When this fraction, in the presence of ATP, is added either at the beginning or at the end of a nucleosomal assembly reaction, it can convert a DNA template consisting of irregularly spaced nucleosomes into a chromatin structure made up of regularly spaced nucleosomes. The nucleosomal repeat length of this chromatin is approximately 165 bp. Chicken histone H1 is able to increase the nucleosomal repeat from 165 to 190 bp in a second step.

**Experimental Procedures**

**Plasmid DNA**—The plasmid used in this investigation was pXbsF201, a 2.9-kb plasmid which carries a 240-bp insert of the X. laevis histone H3.1 gene.

**Purification of Histones H1, H2A, and H2B**—Histone H1 was purified from chicken red blood cells by phosphocellulose chromatography as described in (34) and dialyzed against 0.1 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiotheitol, 10 μg/ml phenylmethylsulfonyl fluoride. The concentration of histone H1 was calculated as described in (35) and checked by Comassie Blue staining using protein standards. Histone H2A and H2B dimers were also purified from chicken red blood cells following the method described in Ref. 22 except for one modification. The concentration of KCl in the sucrose gradient buffer was reduced from 83 to 23 mM.

The 0.2 M NaCl DEAE fraction was obtained from the S-150 as follows. S-150 (20-25 ml), prepared from eight large frogs, was adsorbed onto a 2-ml DEAE-Sephacel column (Pharmacia LKB Biotechnology Inc.) equilibrated with the same buffer. The column was washed with 0.1 M NaCl in buffer A and then eluted with the same buffer containing 0.2 M NaCl. 500-μl fractions were collected. Five fractions with the highest protein content were combined (final protein concentration 2-3 mg/ml) and dialyzed against 0.075 M NaCl in buffer A with 10 mM β-glycerophosphate (or further fractionated with the same buffer containing 0.2 M NaCl). 500-μl fractions were collected.

To isolate the nucleosome spacing activity, the undialyzed 0.2 M NaCl DEAE fraction was loaded onto a 1-ml phosphocellulose P11 column (Whatman) equilibrated with buffer A containing 0.25 M NaCl. The flow-through, which contains N1/N2-(H3,H4), was collected and the column was then washed with the same buffer. Greater than 90% of the total histone H3 and H4 content of the 0.2 M NaCl DEAE fraction can be recovered in the phosphocellulose flow-through fraction. The nucleosome spacing activity was eluted from the column with 0.6 M NaCl in buffer A. Two 500-μl fractions with the highest protein content were pooled (0.2-0.3 mg/ml). This fraction and the flow-through fraction were dialyzed against 0.075 M NaCl in buffer A with 10 mM β-glycerophosphate and stored as aliquots at −70 °C. The 0.6 M NaCl phosphocellulose fraction has been isolated on four separate occasions, from different frogs, and each fraction has given identical results. Running an overloaded sample of the 0.6 M NaCl phosphocellulose fraction on an acid-urea gel revealed no significant amounts of proteins running in the position of histones H1, H2A, or H2B. Protein bands running with histones H3 and H4 have been observed, but the amounts of these proteins would be equivalent to 1/15 to 1/25 of the N1/N2-(H3,H4) added to the chromatin assembly reaction.

**Chromatin Assembly Reaction**—All reactions were carried out at 27 °C for 5 h. Chromatin assembly reactions using S-150 were performed as described previously (37). The extent of reconstitution observed with N1/N2-(H3,H4) purified to different extents (e.g., the 0.2 M DEAE fraction versus the 5 S sucrose fraction) and by different methods (e.g., the 0 S sucrose fraction versus the flow-through fraction), varies at a given protein/DNA ratio. The extent of reconstitution may also vary between different preparations of N1/N2-(H3,H4) isolated by the same method. To compensate for this problem, it was necessary to quantitate the level of N1/N2-(H3,H4) complex required to convert 100 ng of relaxed DNA into supercoiled DNA (22). The addition of 2.0 to 0.8 units of N1/N2-(H3,H4) complex of DNA to an assembly reaction results in the major products of chromatin assembly being templates having 17 to 18 nucleosomes, as judged by two-dimensional gel electrophoresis (29).

**Micrococcal Nuclease Digestion Analysis and Agarose Gel Electrophoresis**—The chromatin was digested with micrococcal nuclease (Boehringer Mannheim) and the DNA fragments were separated by agarose gel electrophoresis. The concentration of proteins was determined using the Bio-Rad protein dye reagent with bovine serum albumin as protein standard.
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RESULTS

Fractionation of the S-150 Extract—The S-150 extract prepared from X. laevis oocytes efficiently assembles plasmid DNA into authentic chromatin. Under the conditions used here, the nucleosomal repeat length of this chromatin is 180 bp (37). Fractionation of the S-150 extract, and the eventual purification of all nucleosomal assembly components, will reveal the mechanism by which regularly spaced nucleosomes are assembled. Substituting Xenopus components with counterparts purified from other vertebrate sources may also help in elucidating the mechanism.

Fig. 1 shows the fractionation scheme used in this investigation to identify and separate a nucleosome spacing activity from core nucleosomal components. In addition, this figure summarizes the results of the investigation. In the oocyte and egg, histones H3 and H4 are complexed with proteins N1 and N2 (26). Immunodepletion of this complex in unfractionated egg extracts inhibits nucleosome assembly on replicating DNA and on nonreplicating plasmid DNA (25). When the S-150 is loaded onto a DEAE column at 0.1 M NaCl, the N1/N2-(H3,H4) complex is retained (22–24). It has been shown recently that all of histones H2A and H2B flow through this column and that histone H2A is composed of three variants (13, 24, 25). In this investigation, we have used single nonvariant H2A and H2B species, purified from chicken red blood cells, to study nucleosome spacing. N1/N2-(H3,H4) is eluted off the DEAE column at 0.2 M NaCl, and when this fraction is mixed with plasmid DNA and purified histones H2A and H2B, chromatin with a nucleosomal repeat length of 165 bp is produced. Addition of histone H1, also purified from chicken red blood cells, to this reaction increases the repeat length to about 190 bp. To investigate the mechanism responsible for generating a regular nucleosomal array with a 165-bp repeat, the 0.2 M NaCl DEAE fraction was subjected to phosphocellulose chromatography. When the flow-through, which contains the N1/N2-(H3,H4) complex, is combined with DNA and with histones H2A and H2B, nucleosomes are assembled but are not regularly spaced. Addition of the 0.6 M NaCl phosphocellulose fraction to this reaction, either at the beginning or at the end of the assembly reaction, regenerates chromatin with a 165-bp repeat. This demonstrates that the 0.6 M phosphocellulose eluate contains a nucleosome spacing activity. The reaction requires ATP. Identical results are obtained when the N1/N2-(H3,H4) complex is isolated by sucrose gradient centrifugation instead of by phosphocellulose chromatography.

Fig. 2A shows a sodium dodecyl sulfate protein gel of the X. laevis oocyte fractions used in this investigation; lane 8 shows the proteins in the 0.6 M NaCl phosphocellulose fraction which contains the nucleosome spacing activity. Because of the requirement for ATP in nucleosome spacing, this fraction was incubated with [γ-32P]ATP, and the labeled proteins were also run on a sodium dodecyl sulfate protein gel (Fig. 2B). Interestingly, a labeled band runs in the position of HMG 17. When these same labeled proteins are run on an acid-urea gel, the labeled band now runs in the region of HMG 14, and not with HMG 17, suggesting that this protein may be of the HMG I/Y class (39, data not shown). Lane 8 also reveals another interesting protein that runs in the same position as HMG 1 (also on an acid-urea gel, data not shown).

FIG. 1. Fractionation of the nucleosome spacing activity and summary of results.

FIG. 2. Protein composition of the fractionated components from oocyte S-150. A, the fractionated components from S-150, isolated as shown in Fig. 1, were analyzed on a 18% sodium dodecyl sulfate-polyacrylamide gel. The gel was double-stained, first with silver and then with Coomassie Blue. Lane 1, molecular weight markers (see "Experimental Procedures" for molecular weights); lane 2, total histones isolated from chicken red blood cells; lane 3, 3 μg of human placenta high mobility group proteins 1, 2, 14, and 17; lane 4, 5.2 μg of S-150c; lane 5, 6.1 μg of the 0.2 M NaCl DEAE fraction; lane 6, 8.4 μg of the phosphocellulose flow-through fraction; lane 7, 2.5 μg of the S sucrose gradient fraction; lane 8, 0.8 μg of the nucleosome spacing fraction. B, the nucleosome spacing fraction was incubated with [γ-32P]ATP for 60 min, as described under "Experimental Procedures," and the reaction mix was loaded onto a 18% sodium dodecyl sulfate-polyacrylamide gel. The gel was then subjected to autoradiography.
This protein could be HMG A, an abundant HMG 1-like protein found in amphibians.

**Loss of Nucleosome Spacing by Fractionation of the S-150**—We have used micrococcal nuclease to compare the structure of chromatin formed using unfraccionated and fractionated S-150 components. At the end of the chromatin assembly reaction, micrococcal nuclease is added and the chromatin is digested for increasing lengths of time. The DNA is purified and subjected to agarose gel electrophoresis. Only early digestion time points are used to determine nucleosomal repeat lengths as more extensive digestion results in the trimming of linker DNA which reduces the true value of the repeat length.

Micrococcal nuclease digestion of chromatin formed after a 5-h incubation between S-150 and plasmid DNA generates a regular and extended nucleosomal ladder of DNA fragments (Fig. 3, lanes 2 and 3). As reported previously, the nucleosomal repeat length for this chromatin is 180 (175-185) bp (37).

FIG. 3. Isolation of N1/N2-(H3,H4) from the S-150 results in the loss of nucleosome spacing. Chromatin was assembled, digested with micrococcal nuclease, and the DNA fragments were purified as described under “Experimental Procedures.” The DNA fragments were analyzed on a 1.5% agarose gel. Chromatin assembled from unfraccionated S-150 and plasmid DNA was digested for 2, 4, 8, 16, and 32 min, lanes 1–5, respectively (0.05 unit of enzyme/µl of reaction). Chromatin assembled from the 0.2 M NaCl DEAE fraction (0.2 DEAE), histones H2A and H2B, and plasmid DNA was digested (0.16 unit/µl reaction) for 1, 2, 4, and 8 min, lanes 6–9, respectively. Chromatin assembled from the 5 S sucrose gradient fraction, histones H2A and H2B, and plasmid DNA was digested (0.03 unit/µl reaction) for 4, 8, 16, and 32 min, lanes 10–13, respectively. Identical reactions were carried out except that the 5 S fraction was replaced by the phosphocellulose (PC) flow-through. The assembly product was digested (0.13 unit/µl reaction) for 2 min, lane 14. m, high molecular weight markers; n, low molecular weight markers (see “Experimental Procedures” for sizes in bp).

Incubation of the 0.2 m NaCl DEAE eluate with histones H2A and H2B and plasmid DNA, under the same conditions, produces a chromatin structure consisting of regularly spaced nucleosomes, but the repeat length decreases to approximately 165 (162–166) bp (Fig. 3, lanes 6 and 7). This indicates that a component or components responsible for increasing the repeat length from 165 to 180 bp must be missing from the reaction.

When N1/N2-(H3,H4), isolated either by sucrose gradient centrifugation (Fig. 3, lane 10) or by phosphocellulose chromatography (Fig. 3, lane 14), is mixed with histones H2A and H2B and DNA, the same number of nucleosomes form as in the reaction receiving the 0.2 m NaCl DEAE eluate, but they are not spaced in a regular manner. This is shown by the loss, at low digestion time points, of an extended nucleosomal periodicity which is replaced by a heterogeneous smear of DNA fragments (compare lanes 10 and 14 with lanes 2 and 3, and with lanes 6 and 7). More extensive digestion of DNA regions between nucleosomes reveals that a few adjacent nucleosomes are closely packed since a limited 145-bp repeat length is observed (lane 13). It is worth noting that, when nucleosomes are spaced in a regular manner, the earliest of digestion time points already display a regular ladder of DNA fragments (Fig. 3, lanes 2 and 6). These results suggest that the 0.2 m NaCl DEAE eluate contains an activity responsible for both organizing nucleosomes into a regular array and setting up a nucleosomal repeat length of 165 bp and that further purification of N1/N2-(H3,H4) from this column fraction results in the loss of the spacing activity.

**Histone H1 Can Increase the Nucleosomal Repeat Length from 165 to 190 bp**—It has been shown that histone H1 and H5 alone can space nucleosomes when a poly[d(A-T)]/poly[d(A-T)] template is used in the presence of polyglutamic acid (11). It has also been shown recently that the addition of excess histone H1 to unfractionated S-150 can significantly change the 180-bp repeat length (37). It is therefore possible that histone H1 or an H1-like protein is present in the S-150 and that this protein is involved in the formation of regularly spaced nucleosomes. In an attempt to reconstitute the nucleosome spacing observed here, chicken histone H1 (1 molecule per 190 bp) was first added to nucleosome assembly reactions containing N1/N2-(H3,H4), histones H2A and H2B, and plasmid DNA. Added histone H1 did not space nucleosomes into a regular array (data not shown). Therefore, at least under the conditions employed here, using physiological histone complexes, histone H1 alone could not function as a nucleosome spacing factor.

Next, we investigated whether histone H1 can increase the nucleosomal repeat length. Identical chromatin assembly reactions were carried out, except that the 0.2 m NaCl DEAE eluate replaced N1/N2-(H3,H4) in the reactions. Fig. 4 shows that histone H1 can increase the nucleosomal repeat length from 165 bp to approximately 190 (187–194) bp (Fig. 4, compare lane 1 with lane 5). The same results were obtained when histone H1 was added 30 min after the start of the assembly reaction (data not shown). This same repeat length is found in red blood cells before the increase in concentration of histone H5 (40). In addition, we found that histone H1 purified from human placenta can increase the repeat length to around 180 bp (data not shown). These results suggest that histone H1 or an H1-like protein may be responsible for producing the final repeat length of 180 bp in unfraccionated S-150. Alternatively, a completely different mechanism may operate in the oocyte and histone H1 can functionally replace this process. These possibilities will be the focus of a separate study.
Identification of a Nucleosome Spacing Activity—Our next major aim was to partially purify the activity responsible for generating chromatin with a 165-bp repeat. When the 0.2 M NaCl DEAE fraction, histones H2A and H2B, and plasmid DNA. Chromatin lacking histone H1 (−H1) was digested with micrococcal nuclease (0.16 unit/μl reaction) for 2, 4, 8, and 16 min, lanes 1–4, respectively. Chromatin assembled in the presence of histone H1 (+H1) was digested (1.00 unit/μl reaction) for 4, 8, 16, and 12 min, lanes 5–8, respectively. m, high molecular weight markers; n, low molecular weight markers.

of other proteins, e.g. bovine serum albumin, could not induce spacing (data not shown).

We investigated whether the nucleosome spacing activity is required during nucleosome formation, or whether it can function when added at the end of the nucleosome assembly reaction. Fig. 5 (lanes 9 and 10) shows that the spacing activity can function when added after the assembly of nucleosomes is complete. Therefore, nucleosome formation and nucleosome spacing can be distinct sequential processes.

Nucleosome Spacing Activity Requires ATP—A number of studies using unfractionated extracts have shown that ATP is required for the assembly of physiologically spaced chromatin (13–15, 18, 21, 30). We examined whether the nucleosome spacing activity identified in the 0.6 M NaCl phosphocellulose fraction requires ATP by performing nucleosome assembly reactions, in the presence of the spacing activity, in which ATP was replaced by the nonhydrolyzable ATP analogue, adenylyl-5’-yl imidodiphosphate (AMP-PNP). Fig. 6 (lanes 4–6) again shows the regular 165-bp ladder obtained when ATP is used in the reaction. In contrast, when AMP-PNP is used instead of ATP (lanes 7–9) the regular ladder is lost (compare lanes 4 and 5 with 7 and 8). This pattern is similar to that obtained when, in the presence of ATP, the

**Fig. 4.** Histone H1 can increase the nucleosomal repeat length from 165 to 190 bp. In the presence or absence of histone H1, chromatin was assembled using the 0.2 M NaCl DEAE fraction, histones H2A and H2B, and plasmid DNA. Chromatin lacking histone H1 (−H1) was digested with micrococcal nuclease (0.16 unit/μl reaction) for 2, 4, 8, and 16 min, lanes 1–4, respectively. Chromatin assembled in the presence of histone H1 (+H1) was digested (1.00 unit/μl reaction) for 4, 8, 16, and 12 min, lanes 5–8, respectively. m, high molecular weight markers; n, low molecular weight markers.

**Fig. 5.** Identification of a nucleosome spacing activity in the 0.6 M NaCl phosphocellulose fraction. Nucleosome assembly reactions were carried out using the 5 S sucrose gradient fraction, histones H2A and H2B, and plasmid DNA. Chromatin formed in the absence (lanes 1–4) or presence (lanes 5–8) of the phosphocellulose fraction was digested (0.03 unit/μl reaction) for 2 min (lanes 1 and 8), 4 min (lanes 2 and 7), 8 min (lanes 3 and 6), and 12 min (lanes 4 and 5). Identical reactions were performed, except that the 0.6 M NaCl phosphocellulose fraction was added at the end of the nucleosome assembly reaction, and the reaction was allowed to proceed for an additional 60 min. This chromatin was digested (0.05 unit/μl reaction) for 4 and 2 min, lanes 9 and 10, respectively. m, high molecular weight markers; n, low molecular weight markers.
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0.6 M NaCl phosphocellulose eluate is omitted from the assembly reaction (lanes 1 and 2). Importantly, the final nucleosome density reached at the end of the assembly reaction was the same whether ATP or AMP-PNP was used (data not shown). Nucleosome spacing is not observed when ATP is replaced with UTP, GTP, or EDTA (data not shown), see “Experimental Procedures”). On the other hand, as has been observed using an unfraccionated oocyte extract (15), nucleosome spacing is observed when ATP is substituted with the hydrolyzable analogue adenosine 5'-O-(thiotriphosphate) (ATP+S) (data not shown). We therefore conclude that the nucleosome spacing activity, identified in the 0.6 M NaCl phosphocellulose eluate, requires ATP to function.

**DISCUSSION**

To investigate the molecular mechanism of nucleosome spacing, we have fractionated a *X. laevis* oocyte extract (S-150) into basic chromatin assembly components that, when combined, produce chromatin with physiologically spaced nucleosomes. The important findings of this study are: 1) chromatin with a nucleosomal repeat length of 190 bp can be assembled in two steps, 2) a novel nucleosome spacing activity, which has been partially purified, can organize nucleosomes into a regular array with a repeat length of 165 bp, 3) ATP is required for this initial spacing step, and 4) histone H1 can increase the nucleosomal repeat length from 165 to 190 bp.

Previous studies have shown that the assembly of nucleosomes 	extit{in vitro} using cellular extracts occurs in two steps (17, 18, 22–28). This can be explained by the observation that, in *X. laevis* oocyte and eggs, the two histone pairs exist in two distinct forms. Histones H3 and H4 are complexed with proteins N1 and N2, while histones H2A and H2B are either in a free form or complexed with nucleoplasmin (24–26). Interestingly, histones H3 and H4 isolated from HeLa cells exhibit chromatographic behavior on a phosphocellulose column similar to that of N1/N2-(H3,H4) suggesting that, in HeLa cells, these histones may also exist as a complex (28). The importance of having histones H3 and H4 in a soluble complex is exemplified by the observation that nucleosomes form efficiently under physiological conditions when these histones are complexed to N1/N2 (22–24, 26), whereas elaborate procedures using nonphysiological conditions are required when purified histones H3 and H4 are used in nucleosome assembly reactions (8–10). The association of histones H3 and H4 with a carrier protein complex may allow the transfer of these histones to DNA to be regulated by the cell. Since the structural core of a nucleosome is the H3/H4 tetramer, the extent and rate of transfer of these histones to newly replicated DNA could determine the rate of nucleosome formation and final nucleosome density. Recently, a nuclear protein purified from HeLa cells, known as CAF-I, has been shown to stimulate the formation of H3/H4 tetramers on newly replicated DNA 	extit{in vitro} (28).

As has been observed with nonphysiological nucleosome assembly systems, nucleosomes assembled from N1/N2-(H3,H4), purified histones H2A and H2B and plasmid DNA are not spaced in a regular manner (22–24, this investigation). Clearly then, other factors are needed to generate an ordered nucleosomal array. We have used phosphocellulose chromatography to identify a novel nucleosome spacing activity, and to separate this activity from N1/N2-(H3,H4). When these two components are combined with histones H2A and H2B, and DNA, chromatin composed of regularly spaced nucleosomes is assembled. The nucleosomal repeat length of this chromatin is approximately 165 bp. The elution properties of the spacing activity on a phosphocellulose column is consistent with the suggestion that at least one component in the nucleosome spacing fraction has DNA binding activity. A number of nonhistone DNA binding proteins (high mobility group proteins) are eluted from a phosphocellulose column at the same salt concentration (39). Indeed, we may have identified at least two such proteins in the spacing fraction. Higher salt concentrations are required to elute off histone H1 (data not shown). Histone H1 appears to be absent in yeast (41), and the nucleosomal repeat length is about 160 bp (42). It is possible, therefore, that the mechanism responsible for generating the 165-bp repeat in *X. laevis* may operate in yeast.

A number of investigations using unfraccionated cellular extracts have demonstrated a requirement for ATP in nucleosome spacing (13–15, 18, 21, 30), but the role of ATP in this process is not known. We show here that the formation of the regular 165-bp nucleosomal repeat requires ATP, suggesting that a phosphorylation step may be involved in generating this chromatin. An attractive working hypothesis is that phosphorylation of a nonhistone protein is necessary to allow it to bind to the nucleosome and induce spacing, or that phosphorylation of a bound nonhistone protein may lead to the weakening of histone-DNA interactions which in turn allows nucleosome spacing. It was recently reported that DNA-dependent phosphorylation, during the very early stages of the chromatin assembly reaction, of an H2A variant in an unfraccionated *X. laevis* oocyte nuclear extract may be involved in nucleosome spacing (30). However, incubation of avian histones H2A and H2B, ± DNA, with the 0.6 M NaCl phosphocellulose spacing fraction in the presence of \(\gamma^{32}P\)
ATP revealed that these histones are not phosphorylated and no additional proteins are phosphorylated in the presence of DNA (in a similar experiment, we also found that histone H1 is not phosphorylated). These results indicate that phosphorylation of H2A or H2B is not needed for nucleosome spacing. It is possible, however, that phosphorylation of the histone H2A variant found in the oocyte may increase the nucleosomal repeat length (see below). Obviously, purification of the component(s) responsible for spacing nucleosomes is required to elucidate the precise mechanism of the spacing process, including the role of ATP in the reaction. It is also possible that ATP may be required at more than one step in the chromatin assembly process (18).

An important finding with regard to the mechanism of nucleosome spacing is the ability of the spacing activity to convert a template consisting of irregularly spaced nucleosomes into a chromatin structure made up of regularly spaced nucleosomes when the spacing fraction is added at the end of a nucleosome assembly reaction. This demonstrates that the two components of chromatin formation, nucleosome assembly and nucleosome spacing, are distinct processes that can be functionally and sequentially separated. Furthermore, the result implies that nucleosomes are not tightly bound to DNA and that the spacing fraction plus ATP can induce nucleosome sliding or reorganize the structure of the histone octamer to convert an irregularly spaced nucleosomal template into a regular array of nucleosomes. Histone H4 present in the N1/N2-(H3,H4) complex is in a diacetylated form (24) and it is possible that this modification, and perhaps other histone modifications, may facilitate nucleosome sliding.

Since histone H1 binds to the nucleosome and associates with linker DNA, it is believed that it plays a role in spacing nucleosomes. However, there is limited direct evidence supporting this hypothesis. Under the physiological conditions used here, histone H1 alone could not space nucleosomes when added to core nucleosome assembly components. Other unsuccessful attempts using physiological conditions and substrates have also been reported (8, 43). We show here that histone H1 can increase the nucleosomal repeat length from 165 to 190 bp and maintain nucleosomes in a regular array. This result suggests that histone H1 may require the spacing activity identified here to generate a regular nucleosomal array. One possible explanation for this requirement is that the final repeat length is the sum of the individual contributions by the spacing activity and histone H1 and that histone H1 requires a regular nucleosomal array to increase the repeat length. The second possibility is that the 165-bp repeat itself is not required for nucleosome spacing by histone H1; it may be the sliding of nucleosomes that is required. These two possibilities may not be mutually exclusive, i.e. the sliding of nucleosomes and the generation of a 165-bp repeat may be important for histone H1 to function. With regard to these possibilities, it was shown recently that histone H1 and H5 can alone space nucleosomes in the presence of polyglutamic acid when a poly[d(A-T)]/poly[d(A-T)] template was used (11). Apparently, under these conditions, nucleosomes can slide apart in the presence of histone H1 and H5 (44). Concerning the mechanism that determines the final repeat length of mature chromatin, this same study showed that the spacing periodicity obtained was determined by the initial nucleosome packing density, i.e. histone H1 or H5 could not space nucleosomes more than the density permitted (11). A similar conclusion was reached using unfractionated S-150 extract (13).

Support for this proposal comes from the observation that replacement of histone H1 with histone H5 in rat sarcoma cells did not alter the nucleosomal repeat length of the chromatin (45). It is possible, then, that the controlling step in determining repeat length is the step that regulates histone deposition during DNA replication (see above).

The nucleosomal repeat length produced by unfraccionated S-150 under the conditions employed here is about 180 bp. Using fractionated S-150 components and histones H2A and H2B purified from chicken red blood cells, chromatin with a repeat length of 165 bp can be assembled. The increase in repeat length induced by histone H1 suggests that a histone H1 or H1-like protein in unfraccionated S-150 may be responsible for producing the final repeat length of 180 bp. However, despite a report identifying histone H1 in oocytes (46), attempts to identify histone H1 in purified assembly products have been unsuccessful (13, 25). Although proteins could “fall off” the chromatin during the long centrifugation step used to isolate the assembly products in the latter study, it is possible that other mechanisms, responsible for generating the 180-bp repeat, may operate in oocytes. It has been shown that phosphorylation of the N-terminal domain of sea urchin histone H2B can inhibit its binding to DNA (47). Increasing the negative charge of the oocyte histone H2A variant by phosphorylation (30) may have a similar effect, and thus may also facilitate nucleosome sliding. A repeat length of 180 bp instead of 165 bp may be achieved if this histone variant is used in place of the avian histone H2A in our fractionated assembly system. This possibility will be tested.

The results of this work and of previous studies on nucleosome assembly (17, 18, 22–28) suggest that the assembly of chromatin in vivo is a sequential process occurring in at least four steps. First, histones H3 and H4 bind to DNA to form a subnucleosomal particle. Next, histones H2A and H2B bind to complete the assembly of the nucleosome. Following this, the spacing activity identified here can reorganize nucleosomes into a regular array and finally histone H1 functions to generate mature chromatin. Fractionation of nascent chromatin at different times after DNA replication has demonstrated that this order of addition of histones occurs in vivo (29). The last two steps of the in vivo sequential process also mimic the increase in nucleosomal repeat length that occurs in vitro (31). The likely involvement of a phosphorylation step in the spacing reaction may provide a mechanism by which this step can be regulated during the S phase of the cell cycle.

When a eukaryotic gene is activated major structural changes in chromatin occur. To understand this process, it is first necessary to establish how DNA is assembled into chromatin. It has been suggested that the sequential binding of core histones to DNA in vivo may facilitate the formation of the transcription complex (3, 22). Since the spacing reaction may involve nucleosome sliding, this stage of the assembly reaction may also facilitate transcription factor binding to DNA, before histone H1 binds to and locks nucleosomes into an inert structure. Through the action of the spacing activity, DNA binding proteins may be able to uncover important DNA elements directly by displacing nucleosomes, or indirectly by specifically positioning adjacent nucleosomes. Potentially, activation of a promoter in the absence of DNA replication may also require a phosphorylation step to allow the necessary disruption of chromatin. By combining this fractionated chromatin assembly system with an in vitro transcription system, these possible mechanisms for activating a gene are currently being examined.

Acknowledgments—We thank Luke Hyman for expert technical assistance. We also thank Horace Drew for providing purified histone H1 and for many helpful discussions.
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