Histones Synthesized for Use in Early Development of *Xenopus laevis* Are Stored as a Complex with Antigenic Properties Similar to Those of the Octamer Core of Nucleosomes

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ABSTRACT Serum of patients with systemic lupus erythematosus (SLE) contains crossreacting autoantibodies which recognize histones in nucleosomes or when they are induced to form octamers in solution in the presence of 2 M NaCl, but not when they are dissociated free in solution at physiological ionic strength. We have found that histones stored in eggs of *Xenopus laevis* for use in rapid nuclear synthesis during early development react with this antibody. This reaction has been observed by radioimmunoassay, inhibition of chromatin assembly by the extracts in the presence of antibody, and, in a preliminary result, by identification of a histone-antibody complex bound to protein A-sepharose. Further evidence that the extract antigen corresponds to the stored histone pool comes from sedimentation and charge fractionation experiments where the chromatin assembly activity and antigen (measured by radioimmunoassay) were found to cofractionate. Because the extract histones are not bound to DNA, our results suggest that they are stored as a soluble complex in a conformation similar or identical to the octameric core of the nucleosome. Our data suggest that the histones in this complex are bound to an anionic factor or factors which presumably replaces the DNA in shielding the positive charges on the histones.

The protein core of the nucleosome is an octameric complex of histones (1–3). In the absence of DNA this complex is unstable at physiological ionic strength (4, 5) though it does form in 2 M NaCl (2, 3). Apparently, the salt mimics the effect of DNA in masking the positive charges on the histones. The pathway of nucleosome assembly in vivo is not known (if indeed there is a single pathway; see reference 6). Specifically, it is not clear whether the octamer forms before the association of histones with DNA or whether the histones add sequentially to the DNA. Recent evidence suggests that H3/H4 are deposited on the DNA before the other histones (7–9), but it is not known whether this sequential addition of the histones is obligatory.

It has recently been found that anionic polypeptides can act as nucleosome assembly factors in vitro. The first of these polypeptides to be described, nucleoplasmin, was purified from eggs of the frog *Xenopus laevis* on the basis of its assembly promoting activity (10). Nucleoplasmin apparently acts by first forming a complex with the histones and then transferring them to DNA (10). It has more recently been reported that polyglutamic acid, which is active as an assembly factor under conditions very similar to those for nucleoplasmin, is capable of organizing the histones into an octamer at physiological ionic strength (11).

Protein crosslinking has until recently provided the only assay for the presence of histone octamers in heterogeneous solutions (2). This technique has the limitation that it measures the stoichiometry of a complex rather than its conformation. Recently, two conformation specific assays for the presence of histone octamers have been developed. These involve the use of either fluorescent probes (12) or a conformation specific antibody (13).

The antibody, a cross-reacting antinuclear antibody (X-ANA), is purified from the sera of certain patients having systemic lupus erythematosus. It binds both to an uncharacterized cell surface antigen of leukocytes and to nucleosomes (13). The antigenic determinant is present both on mononucleosomes and on histone octamers in 2 M NaCl, but not on free histones at physiological ionic strength, on lightly trypsinized mononucleosomes, or on nucleosomes depleted of H2A and H2B (13, 14). The antibody thus apparently provides a probe for the existence of histones in the octameric form.

The state of the histones in the *Xenopus* eggs is of particular interest, as extracts made from these eggs can be used to assemble purified DNA into chromatin with the physiological 200 base pair spacing between nucleosomes (17; also Earnshaw, W. C., and R. A. Laskey, manuscript in preparation). The only

THE JOURNAL OF CELL BIOLOGY · VOLUME 92 MARCH 1982 871-876

other in vitro assembly system where the correct nucleosome spacing is achieved also uses histones stored for use in early development (of *Drosophila*-18). Our results using X-ANA as a probe suggest that the *Xenopus* egg histones are stored as preassembled complexes conformationally similar to the octamer core of the nucleosome.

MATERIALS AND METHODS

Reagents

The following components were purified as previously described: nucleoplasmin (10, 19); topoisomerase I (nicking-closing enzyme [20]); beef kidney histones (20); mononucleosomes for use in the radioimmunoassay (13). Immunospecific isolation of X-ANA from insoluble polynucleosomes has been described (13). *Xenopus laevis* egg homogenates (17) were extracted with freon (1, 1, 2-trichlorotrifluoroethane) to remove yolk proteins (10). Normal human serum was the gift of S. Sachs (MRC Molecular Biology Laboratory), and immunoglobulin was partly purified from it by two precipitations with 40% ammonium sulphate. ^{125}I-IgA myeloma protein was the gift of B. Bogen (University of Tromsø) and was used as a 7–8 s marker on sucrose gradients.

Sucrose Gradients

Sucrose gradients of egg extracts were run as described (22). Fractions were tested for ability to assemble 3H-SV40 DNA into nucleosomes as described (10, 22), except that 1 μl of topoisomerase I was added to each incubation to relax the DNA which was supercoiled when received as a gift from R. M. Harland (MRC Molecular Biology Laboratory).

Radioimmunoassay (RIA)

The RIA for X-ANA has been previously described (13).

Effect of X-ANA on Nucleosome Core Assembly by Extracts

0.8 μg of X-ANA was incubated overnight at 4°C with 20 μl of extract in the presence of 0.1 mM PMSF and 10 U/ml Trasylol (Aprotinin; Mobay Chemical Co., FBA Pharmaceuticals, New York). In control experiments the X-ANA was replaced by 8 μg of partly purified normal human IgG. In the morning, 0.4 μg of DNA which had been ^{32}P-labeled by nick translation (23) and had free label removed by centrifugation through Sephadex G-25 was added. After incubation at room temperature for 2–3 h, the assembly was stopped by addition of an equal volume of a solution of 1% SDS, 5 mM EDTA, 10 mM Tris-HCl pH 7.6, 0.01% Bromophenol Blue, 0.01% Xylene Cyanol Blue, followed by deproteinization with chloroform:isoamyl alcohol (24:1 vol/vol). The DNA was electrophoresed for 3–4 h in a 1.25% Agarose gel in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 2 mM EDTA pH 8.3). The gel was dried by squashing between layers of porous paper held by glass plates and autoradiographed on Kodak RP X-Omat film.

Incubation with DEAE or CM Cellulose

25 μl of extract was mixed with 20 μl of cellulose slurry (DE25 or CM 25 swollen in 100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.6), incubated at room temperature for 5 min, and the cellulose was removed by centrifugation. The supernatant was removed and use for assembly as described above.

Isolation of Antigen-Antibody Complexes by Staphylococcus Protein A-Sepharose Binding

100 μl of antigen plus phenylmethylsulfonyl fluoride (PMSF) to 0.1 mM final concentration was incubated with 1.8 μg of purified X-ANA in phosphate buffered saline (PBS) (containing 10 mg bovine serum albumin (BSA)/ml) overnight at 4°C. In controls, either antibody was omitted and 50 μl of BSA at 10 mg/ml was substituted, or X-ANA was replaced with 55 μg of normal human IgG (partly purified as described above). In the morning, 50 μl of a 50% slurry of protein A-sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden; CL-4B) was placed in a plastic tube with 50 μl of BSA (100 mg/ml in PBS – 125 mM NaCl, 25 mM $NaPO_4$ pH 7.2), and then 500 μl of PBS was added. The sepharose was centrifuged in a bench-top centrifuge, the supernatant removed, and the pellet washed once more with 500 μl of PBS. The antigen:antibody mixture was added to the washed sepharose, mixed, and incubated for 30 min at 4°C. The sepharose was then washed twice with 500 μl of PBS, all liquid was removed with a drawn-out micropipette, and 50 μl of SDS sample buffer ([24] plus 140 mM β-mercaptoethanol) was added. After boiling for 5 min, the sample was electrophoresed through an 18% acrylamide gel in the presence of SDS (24). For the experiment shown in Fig. 5 the extract had been concentrated fivefold in a Minicon B15 (Amicon Corp., Lexington, MA). Selective immunoprecipitation of extract histones by X-ANA was also observed using unconcentrated extracts, though the bands on gels were fainter.

RESULTS

Detection of X-ANA Antigen in Egg Extracts

Xenopus egg extracts inhibited binding of X-ANA to nucleosomes on the walls of plastic tubes (Fig. 1), indicating that the extracts contained an antigen recognized by X-ANA. From the known concentration of the mononucleosome standard we could calculate that the concentration of antigen was 50–100 μg/ml. This value was similar to the concentration of stored histones in our extracts which had been estimated from previous work to range roughly between 30 and 120 μg/ml (15–17; also unpublished observations). The question immediately arose whether the antigen in the extract which bound X-ANA was chromatin. Each egg should contain ~31 pg of chromatin (assuming a diploid complement of 6 pg and ~25 pg of amplified ribosomal chromatin [25]), giving rise to an approximate maximum chromatin histone concentration of 0.031 μg/ml in the extract. This is at least 1,600-fold lower than the concentration of antigen measured in the RIA. Furthermore, this should be an overestimate, because the bulk of the endogenous chromatin should have been removed by the procedure used for making the extract.

In a control experiment, we asked whether addition of DNA to an extract had any effect on the apparent concentration of antigen. The results of these experiments varied slightly, but the apparent increase in antigen occurring as a result of nucleosome assembly after DNA addition was never more than twofold, and in several experiments no difference was detected (see Fig. 4). The exogenous DNA would rapidly have been assembled into chromatin (17) and, because the histones in chromatin are good antigens for X-ANA, these results suggest that the majority of the antigen in the egg is stored in a form not distinguishable from nucleosomes by our RIA.

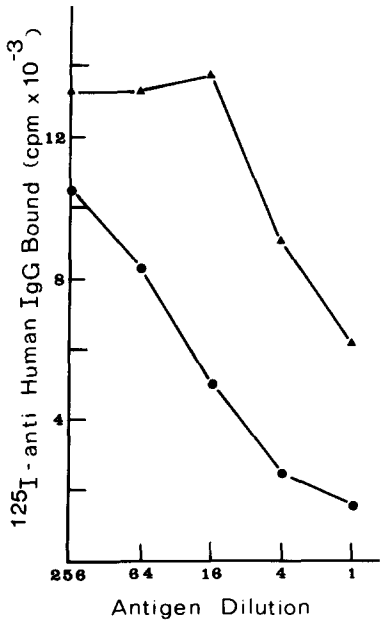

FIGURE 1 RIA of *Xenopus* egg extract. Details of the assay are as previously described (13). Note that a positive interaction of the test antigen with the X-ANA prevents the antibody from binding to a tube which has been coated with polynucleosomes, resulting in decreased binding by a ^{125}I-labeled anti-human second antibody. The starting concentration of the mononucleosome control was 1 mg/ml. (●) Mononucleosomes; (▲) extract.

Preliminary Characterization of the Extract Antigen

Because X-ANA exhibit dual specificity for histones and a plasma membrane antigen, it was necessary to demonstrate that the antigen we detected in the extract by RIA was histone and not related to the crossreacting membrane component. Our conclusion is based on results of a number of types of experiments.

First, the antigen sediments on sucrose gradients at 7–8 s as shown in Fig. 2*a*. From the figure, it can be seen that the peak of antigenicity (maximum inhibition of binding of X-ANA to solid phase-bound polynucleosomes) occurred in fraction 13 of this gradient. When each gradient fraction was tested for its ability to promote nucleosome assembly on added DNA, the peak of assembly activity was found in fractions 12 and 13 (Fig. 2*b*). It therefore appears that the antigenic activity co-sediments with the extract components responsible for assembly (i.e. the stored histones).

Second, we found that X-ANA could specifically inhibit nucleosome assembly by egg extracts (Fig. 3, *track 1*). Partly purified IgG from normal human serum had little effect on nucleosome assembly by the extracts (Fig. 3, *track 2*). That the inhibition by X-ANA was correlated with a removal of histones from the extract was suggested by the observation that, upon addition of purified bovine core histone to an inactivated extract, some nucleosome assembly occurs (data not shown).

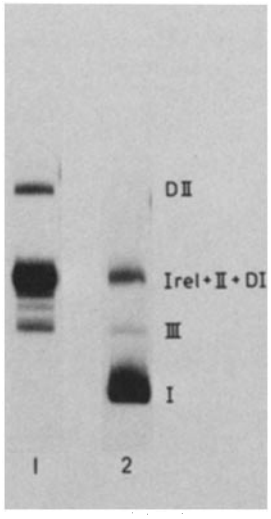

FIGURE 3 Inhibition of nucleosome assembly in egg extracts by X-ANA. The overnight preincubations were: track *1*, extract plus X-ANA; track *2*, extract plus partly purified IgG from normal human serum. The DNA forms indicated are I-monomer supercoiled (assembled), III-monomer linear, Irel-monomer relaxed (unassembled), II-monomer nicked. The figure is somewhat complicated by the presence of pBR322 dimer contaminating the DNA. The dimer-derived bands have been labeled DI and DII, and these correspond to the monomer labeling given above (except that the DII region probably contains DIrel as well). The few bands of intermediate superhelicity seen in track *1* reflect a very low level of assembly, since this low percentage gel (1.25%) does not resolve higher superhelix densities.

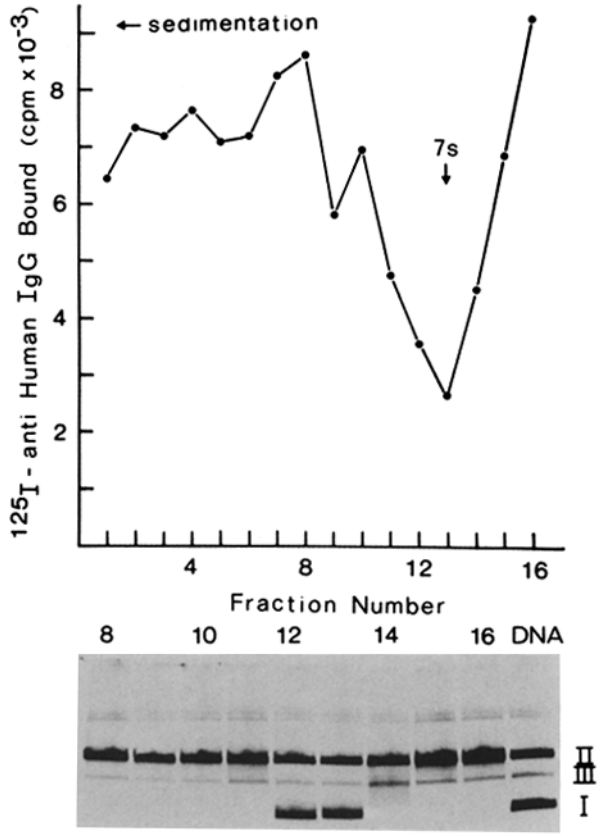

FIGURE 2 *(a)* RIA of each fraction of a 10–30% sucrose gradient of egg extract run as previously described (22).

(b) Assay of each gradient fraction for the ability to assemble ^{3}H-SV40 DNA (labeled in vivo) into nucleosomes. Form II (nicked), form III (linear), and form I (supercoiled – indicating nucleosome assembly) are labeled. Note that form II DNA runs at its characteristic position regardless of whether or not it had been assembled into nucleosomes. The assay thus underestimates the fraction of the population which had assembled. The track at the right labeled DNA shows the input DNA (note that since nicking-closing enzyme was added to the DNA before mixing with the gradient fractions, the form I DNA in this track was converted to form I relaxed before assembly).

The above experiments suggest strongly that the antigen which X-ANA recognises in the egg extracts is the stored histone and further suggest that this histone is stored as a soluble complex similar or identical to the octameric core of the nucleosome. However, histones alone do not form octamers at physiological ionic strength. Our results imply, therefore, that some extract component must be shielding the charges on the histones. This could be either a macromolecular factor bound to the histones (such as nucleoplasmin) or it may be due to extensive modifications of the histones.

The experiment of Fig. 4 shows that the predicted charge shielding does occur. An extract was batch-treated with either CM-cellulose (negatively charged) or DEAE-cellulose (positively charged). As the figure shows, the antigen behaves as though its net charge is negative (i.e., it binds to DEAE and not to CM). In a control experiment (Fig. 4, insert) the ability of the extract to assemble nucleosomes can be shown to have similar anomalous charge properties. The anomalous apparent negative charge is also found for histones which have been bound to purified nucleoplasmin in vitro (20).

As an attempt to further characterize the extract antigen bound by X-ANA, immune complexes were precipitated by their affinity for Staphylococcal protein A as shown in Fig. 5. Because X-ANA has a low binding affinity, washes were performed at physiological ionic strength and we observed a substantial amount of binding to the protein A-sepharose in the controls where BSA or normal human IgG was used in place of X-ANA. While this nonspecific binding complicates the gel patterns, it does not prevent us from observing that X-ANA appears to selectively enhance the binding of at least four proteins. Three of these have the mobility of H3, H2B, and H4, respectively. In addition to the histones, a protein of higher molecular weight is also bound specifically in the presence of X-ANA. This protein has a mobility corresponding to

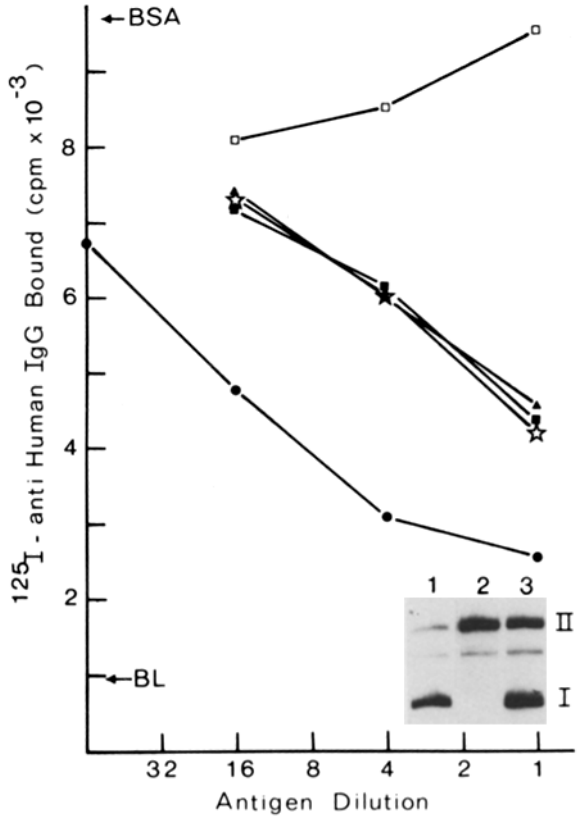

FIGURE 4 The extract histone complex has a net negative charge. RIA was as in Fig. 1. In each case, 500 μl of extract was incubated with 500 μl of cellulose slurry. Where DNA was added, this was 50 μg of pBR322 form I. Note that normally histones bind to CM-cellulose which is negatively charged under these conditions (20). The arrow marked *BSA* marks the counts bound when BSA was used as antigen in the overnight incubation with X-ANA. The arrow marked BL indicates the counts bound when X-ANA was omitted. (●) Mononucleosomes, (▲) extract plus DNA, (□) extract plus DEAE cellulose plus DNA, (■) extract plus CM cellulose, (★) extract plus CM cellulose plus DNA. (*inset*) Effect of DEAE and CM-cellulose adsorption on assembly by egg extracts. Track *1*, untreated extract; track *2*, extract adsorbed with DEAE-cellulose; track *3*, extract adsorbed with CM-cellulose. Experimental details are given in Materials and Methods.

a M_r = 180,000. The large number of proteins which bound nonspecifically to protein A-sepharose could have prevented us from detecting other differences between the control and experimental tracks. Four is therefore a minimum estimate for the number of protein species which were bound by X-ANA. When a control was done using BSA rather than normal human serum, the only differences between the two tracks were these four bands (Fig. 5). Otherwise the control and experimental tracks were remarkably similar.

Given the fact that *Xenopus* chromatin does contain a normal ratio of the four core histones, we were initially troubled by the apparent lack of immunoprecipitation of H2A. That the absence of a typical H2A in eggs is real and not an artifact of the immunoprecipitation is suggested by the data of Fig. 5, *track 7*. When an extract was chromatographed on DEAE in a buffer containing 0.1 M NaCl, 1 mM EDTA, and 20 mM Tris pH 7.5, the histones were retained as predicted by Fig. 4. Upon washing the column with a salt gradient of 0.1–0.5 M NaCl, a sharp peak of assembly activity eluted at ~0.13 M NaCl (Earnshaw, unpublished observations). *Track 7* shows a

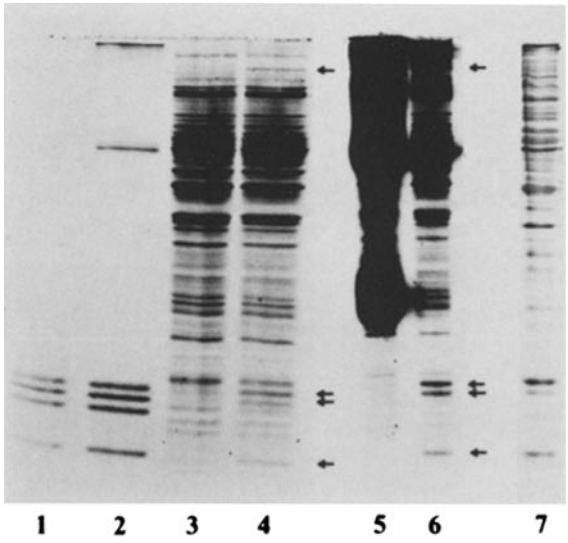

FIGURE 5 Absorption of immune complexes to protein A-sepharose. Experimental details are given in Materials and Methods. Track *1*, mononucleosomes plus BSA; track *2*, mononucleosomes plus X-ANA; track *3*, 5 times concentrated extract plus BSA; track *4*, 5 times concentrated extract plus X-ANA; track *5*, 5 times concentrated extract plus partly purified normal human IgG; track *6*, 5 times concentrated extract plus X-ANA. Tracks *5* and *6* are printed so as to show clearly the difference in the histone region. Bands which are selectively precipitated are indicated with arrows. They are (from top to bottom) M_r 180,000 protein, H3, H2B, and H4. Track *7* shows a fraction from a DEAE cellulose column run of total extract. This fraction was highly active in assembly using the supercoiling assay. Note that histone H2A is not observed at its normal position.

gel of this active fraction. H2A of normal mobility was not observed.

DISCUSSION

Each *Xenopus* egg contains a stockpile of ~20,000 diploid equivalents of histone (15, 16). That the stockpiled histones are stored as a complex is suggested by three separate lines of evidence: sedimentation on sucrose gradients (10); chromatography on DEAE cellulose; and binding to X-ANA. This evidence is largely independent of the histone conformation because in the first two cases the histones were detected using then nucleosome core assembly assay. In this paper we also show, however, that X-ANA recognize an antigen with the sedimentation and charge properties of the histone complex. Additionally, X-ANA specifically inhibit chromatin assembly by the egg extracts. The fact that X-ANA recognize the complex implies that the histones are in a conformation antigenically similar to that of the octamer core of the nucleosome (14).

Our preliminary characterization of the stored histones by acrylamide gel electrophoresis of immune complexes is consistent with the presence of histone octamers, because H3, H2B, and H4 were found to bind to X-ANA. It is intriguing that H2A was seen neither in the immune complexes nor in complexes partly purified by an independent procedure (DEAE cellulose chromatography). It is possible that the extract H2A is extremely sensitive to an endogenous protease, but this is unlikely as extracts may be incubated overnight at either 4° or 20°C without losing their ability to assemble nucleosomes with the physiological 200 base pair spacing (Earnshaw, unpublished observations). Also, exogenous H2A may be incubated overnight in extracts at 4°C without being degraded (data not

shown). Another possibility is that H2A is loosely attached to the histone complex and dissociates during the immunoprecipitation or during binding to DEAE. This cannot be ruled out by our data but is rendered unlikely by the fact that normal H2A is not seen when extract proteins are assembled onto DNA cellulose (Earnshaw, unpublished observations). We favor the possibility that the extract H2A is coupled to another protein, analogous to the formation of the H2A-ubiquitin conjugate A24 (27, 28). In fact, when extract proteins are assembled onto DNA cellulose directly, a protein with the mobility of A24 is observed in amounts equimolar with the three observed histones. Further experiments in progress should demonstrate whether or not this protein is A24. In the gel shown in Fig. 5, enhanced binding of a protein in the A24 region of the gel was not observed, and it is tempting to speculate that the M_r = 180,000 protein represents H2A or A24 conjugated to another extract protein and possibly migrating atypically in these experiments. That H2A is present but in a "hidden" form is suggested by the earlier result that X-ANA require the presence of H2A and H2B to recognize the antigenic determinant (14), and also by the observation that *Xenopus* extracts are active in assembling physiologically spaced nucleosomes. Further characterization of the stored histone complex should result in the explanation of this puzzling observation.

It might be argued that the use of X-ANA as a conformational probe is complicated by the fact that the serum of lupus patients contains a wide range of antinuclear antibodies. This objection should be largely answered by the precaution of obtaining highly purified X-ANA, taking advantage of its binding to both polynucleosomes and leucocytes (13). We therefore feel that purified X-ANA are likely to exhibit a rather limited range of specificities. Furthermore, we have been able to confirm the existence of a histone complex with an unusual electrophoretic profile both by immunoprecipitation with X-ANA and by DEAE cellulose chromatography.

Histones are highly positively charged and do not normally form octamers in the absence of DNA under physiological conditions. Such octamers have only previously been observed under special conditions in vitro-in solutions of high ionic strength (2 M NaCl [2, 3]), or when the histones are complexed with synthetic polyanions (11). For the *Xenopus* histones to be stored as complexes in vivo, the positive charges must be shielded either as a result of extensive postsynthetic modification or by formation of a complex with a macromolecular polyanion (or a number of smaller anionic molecules). The histone octamer sediments at 4–5.2 s (3). The observation that the extract histones sediment at 7–8 s on sucrose gradients (10) together with their anomalous apparent charge properties supports the notion that they are bound to an anionic factor.

The identity of this putative factor is not known. Nucleoplasmin, an anionic protein purified from *Xenopus* eggs, has been previously shown to bind histones and to transfer them to DNA, forming nucleosome cores (10, 20). We have found that nucleoplasmin:histone complexes assembled in vitro from purified components are not recognized by X-ANA, whereas the nucleosome cores, assembled when DNA is added to these complexes, are recognized (data not shown). This experiment is not conclusive, because the stored histones are highly modified (27) and might require these modifications to enter the octamerlike conformation upon binding to nucleoplasmin, but the result does suggest that the in vitro and in vivo complexes are conformationally different and that therefore nucleoplasmin is not the histone binding factor in vivo. This idea is supported by data showing that antinucleoplasmin antibodies do not coprecipitate histones from oocyte extracts (30), and by the observation that the apparent size of nucleoplasmin from extracts does not alter after removal of the histones (Laskey and Honda, unpublished observations). Therefore, both the role of nucleoplasmin and the identity of the histone binding factor in vivo remain as topics for further investigation.

Our findings imply that the stored histones add to DNA as octamers rather than as isolated histone pairs. This contrasts with other recent results (7–9) which have demonstrated the separate addition of H3/H4 and H2A/H2B to newly replicated DNA in a number of cultured cell lines. It is possible that the existence of a large histone pool in the *Xenopus* egg necessitates a strategy of chromatin assembly which is different from that adopted by somatic cells, where the synthesis of histone and the synthesis of DNA are tightly coupled. The shielding of the histone charge which is concomitant with formation of the complex in the egg might be necessary to prevent random electrostatic interactions from interfering with the metabolism of the early embryo.

We thank Jim Paulson for valuable discussions and Ron Laskey and Stu Weisbrod for comments on the manuscript.

W. C. Earnshaw was a NATO postdoctoral fellow. This work was initiated during a vist to Tromsø which was supported by an EMBO Short-Term Fellowship.

Received for publication 2 November 1981, and in revised form 4 December 1981.

Note Added in Proof: Recently J. Kleinschmidt and W. W. Franke (German Cancer Research Center, Heidelberg) have isolated a stored histone complex from *Xenopus* oocyte nuclei (manuscript submitted for publication). Their partly purified complex contains H3, H4, and a high molecular weight acidic protein, as well as lesser and variable amounts of H2b. H2a of normal mobility was not detected in the complex. These results are similar to ours, both reported above and unpublished (though their interpretation may differ in detail). We therefore emphasize that while the stored histone complex does share conformational determinants with nucleosomes, it must also show some differences from the canonical octamer composition. Both our results and those of Kleinschmidt and Franke suggest that pathways of chromatin assembly in vivo (at least during early development) may be more complex than previous studies of nucleosome core assembly in vitro have suggested (6).

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