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Nuclear assembly is independent of linker histones

(nucleus/chromatin/*Xenopus*)

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ABSTRACT **The role of linker histones in the assembly of functional nuclei was examined with the use of a cell-free extract of *Xenopus* eggs that transforms condensed sperm chromatin into DNA-replication-competent pronuclei. When linker histones were removed from the extract, the resultant pronuclei were indistinguishable from those formed in the complete extract. The assembly of functional nuclear membrane, nuclear lamina, and prereplication centers allowed identical DNA replication efficiencies. Thus, linker histones are not required for the assembly of morphologically normal nuclei capable of DNA replication.**

Nuclear assembly occurs on a DNA substrate (1). DNA is progressively folded into increasingly compact chromatin structures within the chromosome (2, 3). The nuclear lamina and nuclear membrane assemble upon the surface of chromatin (4–6). Membrane assembly allows directed nuclear import (7) and capacitates DNA replication which occurs at discrete sites (factories) attached to a nuclear skeleton (8, 9). The biochemical basis for the attachment of the nuclear lamina or nuclear membrane to chromosomes and their roles in the assembly or activity of replication factories are unknown. Presumably, receptors exist within the lamina or membrane that recognize structural features within the chromosome.

The basic repeating subunit of the chromosome is the nucleosome, which contains a variable length of DNA (≈165–220 bp) and all of the histones. A histone octamer (H2A, H2B, H3, H4)$_2$ is wrapped by 146 bp of DNA in 1.75 superhelical turns in the nucleosome core. The fifth linker histone (either histone B4, H1, or H1° in *Xenopus*) interacts with the linker DNA between adjacent nucleosome cores. Folding of nucleosomal arrays into the 30-nm chromatin fiber is stabilized by linker histones (10). However, the relationship between the presence of linker histones in chromatin, stabilization of the chromatin fiber, and further condensation of the chromosome is currently controversial (11, 12). For example, chromosome condensation occurs in *Xenopus* mitotic extracts deficient in histone H1 (13), whereas, the nuclear scaffold or skeleton has been proposed to function in part by it being a major site of histone H1 association (14, 15). The assembly of the chromatin fiber is typically viewed as a prerequisite for the assembly of chromatin loops and subsequent formation of chromatin domains (16, 17). The assembly of folded nucleosomal arrays into chromatin fiber structures dependent on linker histones might also facilitate recognition by chromatin receptors associated with the nuclear lamina or nuclear membrane (4).

Here we make use of *Xenopus* egg extracts to examine the role of linker histones in the assembly of a functional nucleus (2, 18). These extracts contain a single linker histone, B4 (19–21). This protein has 30% amino acid sequence identity with somatic histone H1 and is the only linker histone normally present in sperm pronuclei (20, 21). Histone B4 is also the major linker histone of early embryonic chromatin (21–23). In these experiments we selectively deplete histone B4 from *Xenopus* egg extracts and examine the consequences for nuclear structure and function.

MATERIALS AND METHODS

***Xenopus laevis* Egg Extracts.** Fractionated S-phase extracts were prepared and stored according to methods detailed elsewhere (24) except that Hepes was omitted from the egg lysis buffer. This protocol separates crude *X. laevis* egg lysates into several fractions, two of which are required for nuclear assembly reactions in reconstituted extracts: a cytosolic and a membrane fraction. The clarified cytosol is sufficient to decondense sperm chromatin itself. Both the membrane and cytosolic fractions are required for the formation of replication-competent nuclei with enclosed nuclear envelopes. Nuclei (generally 10^7 nuclei in 100 μl of extract) were concentrated by centrifugation at 3000 × *g*. Demembranated sperm chromatin was also prepared exactly as described (24).

Assays for the Functions of Reconstituted Nuclei. In all experiments, B4-depleted or untreated cytosol was supplemented with 20 mM creatine phosphate, creatine kinase at 50 μg/ml, and 2 mM ATP. Reconstituted nuclear assembly reactions were prepared as previously described (24) and incubated at room temperature for the times indicated in the figure legends. Sperm chromatin was added at the final concentrations indicated in the figure legends. If replication was also to be monitored in the experiment, 1.0 μCi (1 Ci = 37 GBq) of [α-^{32}P]dCTP was added per 10-μl final reaction volume prior to the start of the incubation. For experiments that utilized extracts reconstituted with both cytosol and membranes (see Figs. 2 and 3), 1.5 μl of the membranes was added to the reaction per 10 μl of clarified cytosol. In these cases, nuclear assembly was monitored visually by fluorescence microscopy with the DNA dye Hoechst 33258 and by phase-contrast microscopy. In order to assay nuclear transport in Fig. 2*C*, a fluorescent transport substrate consisting of a rhodamine-labeled simian virus 40 large tumor antigen nuclear localization signal peptide coupled to human serum albumin (25) was added to nuclei at a concentration of approximately 10 μg/ml. Transport substrate was added 2 h after the start of the reaction. Thirty minutes after the addition of substrate, the samples were mixed on slides with an equal volume of fixative (50 mM sucrose/100 mM KCl/1 mM $MgCl_2$/10 mM Hepes·KOH, pH 7.7/3.7% formaldehyde/Hoechst 33258 at 10 μg/ml, and covered with a coverglass. Photographs were taken of the fixed samples through a Zeiss Axiophot microscope with Kodak Tmax 3200 film. DNA replication was assayed after 4 h of incubation at room

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Abbreviations: RPA, replication protein A; BSA, bovine serum albumin.

temperature by removing 5-μl aliquots of the nuclear assembly reaction mixture and treating them as described (24).

Antibodies to B4 and Immunodepletion of Egg Extracts. Affinity-purified antibodies to the histone B4 protein were produced from the serum of rabbits immunized with bacterially expressed B4 protein as described previously (22). Western blot analysis revealed that there is essentially no detectable B4 protein or other linker histones in the membrane components of the reconstituted extracts or in our preparations of demembranated sperm chromatin (ref. 20; see Fig. 1). Immunodepletion of the B4 protein from clarified cytosol was achieved according to the methods described previously (26). Nuclei were assembled as described in mock- and B4-depleted cytosol and membranes.

In order to confirm immunodepletion of the B4 protein, different amounts of depleted cytosol or pronuclei were electrophoresed through an SDS/18% polyacrylamide gel. The proteins were transferred from the gel to polyvinylidene fluoride (PVDF) membranes, which were stained with India ink to assess equivalency of extract loading onto the gel and efficiency of protein transfer to the membranes. The membranes were then incubated with anti-B4 antibodies, which were subsequently visualized by using an ECL detection protocol (Amersham).

Immunofluorescence. The immunostaining protocol was a variation of the published method (27). Sperm nuclei (2000 nuclei per μl) were allowed to form for 2 h under standard assembly conditions in extracts reconstituted with nuclear membranes plus either B4-depleted or mock-treated cytosol (see Fig. 2). Sperm chromatin (4000 nuclei per μl) was allowed to decondense for 1 h in clarified cytosol supplemented with an ATP-regenerating system (Fig. 3). After this incubation, the samples were mixed with an equal volume of fixative lacking the Hoechst 33258 dye and incubated at room temperature for 10 min. Fixation was terminated by the addition of 18 vol of phosphate-buffered saline (PBS; 125 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4) plus 3% bovine serum albumin (BSA). The samples were sedimented onto coverslips through PBS plus 25% glycerol at 1000 × *g* for 3.5 min. The coverslips were incubated once with PBS plus 3% BSA, followed by three washes with PBS plus 0.1% Tween 20. The samples were incubated overnight with primary antibodies in a solution of PBS, 1.5% BSA, and 0.05% Tween 20. The anti-B4 antibody preparation employed was an affinity-purified polyclonal rabbit antibody, which was used at a concentration of 0.6 μg/ml. The anti-replication protein A (RPA) antibody preparation was a polyclonal rabbit serum raised against purified *X. laevis* RPA (28) (the kind gift of F. Fang and J. W. Newport, University of California, San Diego). The antiserum was used at a dilution of 1:200, and there was no significant background observed when preimmune serum from the same rabbit was used with a similar protocol (data not shown). The coverslips were washed three or four times with PBS/0.1% Tween 20 and incubated for 1–2 h with a rhodamine-labeled polyclonal mouse anti-rabbit secondary antibody (Pierce) at a concentration of 6.2 μg of protein per ml of PBS, 1.5% BSA, 0.05% Tween 20, and Hoechst 33258 at 10 μg/ml. The samples were washed four or five times with PBS/0.1% Tween 20, mounted onto slides, and sealed before viewing. Photographs of the samples were taken through a Zeiss Axiophot microscope equipped with the appropriate filters for viewing rhodamine and Hoechst 33258 on Kodak Tmax 3200 film.

The staining of nuclei (Fig. 2) or decondensed sperm chromatin (Fig. 3) formed in the presence or absence of the B4 proteins showed clear differences in the levels of immunofluorescence when we used affinity-purified anti-B4 polyclonal antibodies as the primary antibody. The B4-containing samples were uniformly bright while the depleted samples showed staining similar to that seen with rhodamine-labeled secondary antibodies alone. To show this difference clearly, we mixed the B4-depleted and nondepleted samples together after fixation, added BSA to stop the fixation reaction, and sedimented them onto coverslips for staining.

RESULTS AND DISCUSSION

Egg extracts were depleted of histone B4 by using specific antibodies (Fig. 1; refs. 20 and 22). *Xenopus* sperm chromatin was decondensed in the depleted and control extracts (Fig. 1; ref. 20). *Xenopus* sperm chromatin contains no histone H1 or B4 (20–23). The decondensed sperm chromatin was assembled into pronuclei in both B4-containing and -deficient extracts. Histone B4 was found to be depleted in both the extracts and assembled nuclei by >95% (Fig. 1 and densitometric scanning data not shown). This indicates that extract depletion also leads to chromatin deficient in histone B4 (Fig. 1, lanes 9–14). Nuclei assembled in the B4-deficient extracts have a nuclear lamina (not shown) and a nuclear membrane

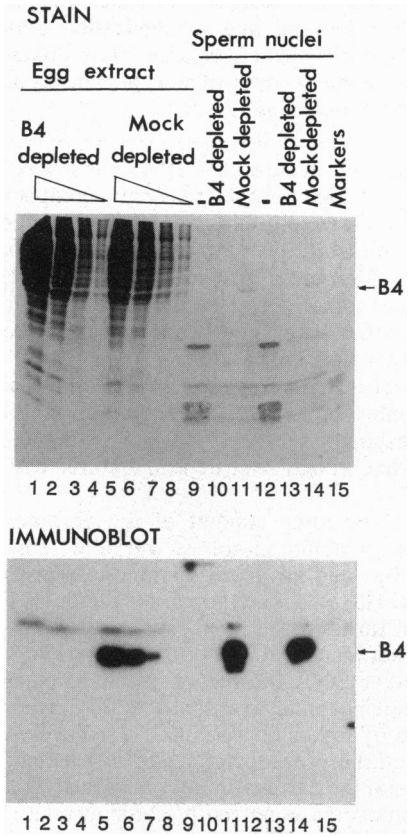

FIG. 1. Immunodepletion of the B4 protein from egg extract and nuclei. Clarified cytosol was immunodepleted of B4 protein. Samples were resolved on an SDS/18% polyacrylamide gel and either stained with Coomassie blue (*Upper*, Stain) or transferred to a membrane (*Lower*, Immunoblot). To confirm that the extract had been depleted of B4, we analyzed an immunoblot of increasing amounts of the mock- and B4-depleted cytosol exposed to affinity-purified anti-B4 antibodies. Lanes 1–4 and 5–8 contain 20, 10, 2, and 0.5 μl of B4-depleted or mock-depleted cytosol, respectively. Sperm chromatin was allowed to form nuclei at room temperature in extracts reconstituted with membranes and either B4-depleted or mock-depleted cytosol (2000 nuclei per μl). The nuclei were concentrated by centrifugation after 2 h. Lanes 9 and 12 contain proteins found in sperm chromatin (2.5 μg of DNA per lane), lanes 10 and 13 contain proteins found in pronuclei (2.5 μg of DNA per lane) reconstituted in B4-depleted cytosol, and lanes 11 and 14 contain proteins found in pronuclei (2.5 μg of DNA per lane) reconstituted in mock-depleted cytosol. The position of B4 is indicated in both panels. Lanes 9–11 and 12–14 show results from two separate extract preparations. Lane 15 shows marker chicken erythrocyte core histones.

(Fig. 2*C*) capable of directed nuclear import (Fig. 2*C*). Control experiments confirmed that nuclear import was active—for example, uptake of the rhodamine-labeled substrate was temperature sensitive, with no import occurring at 4°C (not shown). In addition, import was blocked by wheat germ agglutinin (WGA), indicating that it occurs via the nuclear pore (not shown). In all respects histone B4-deficient nuclei were morphologically and functionally identical to controls. The assembly of nuclei that are deficient in linker histones demonstrates that the putative chromatin receptors associated with the nuclear lamina or nuclear membrane do not recognize structures dependent upon linker histones for their formation.

The rate of sperm chromatin decondensation in B4-deficient cytosol and the rate of assembly of B4-deficient pronuclei were also identical to those in mock-depleted extracts (Fig. 2*B*). Since nuclear volume is unchanged in the presence or absence of linker histone, the compaction of DNA within chromatin necessary to allow accommodation within the nucleus can be achieved without linker histones. DNA is highly compacted within *Xenopus* sperm chromatin and it should be noted that the level of compaction of DNA actually decreases following the assembly of pronuclei.

We next examined whether the linker-histone-deficient nuclei were capable of more complex functions such as the initiation of DNA replication. A prerequisite for chromosomal replication is the assembly of prereplication centers within decondensing sperm nuclei (29). These centers form before the nuclear lamina or nuclear membrane is assembled. However, an intact nuclear membrane and nuclear lamina are both required for the initiation of replication (2, 30–32). Prereplication centers can be immunolocalized with antibodies against RPA (29). RPA has a role in the generation of single-stranded DNA prior to the initiation of DNA replication *per se* (33–35). We decondensed sperm chromatin in histone B4-depleted and control high-speed egg extracts (Fig. 3*A*) that were deficient in nuclear membranes. Under these conditions complete pronuclear formation is arrested after

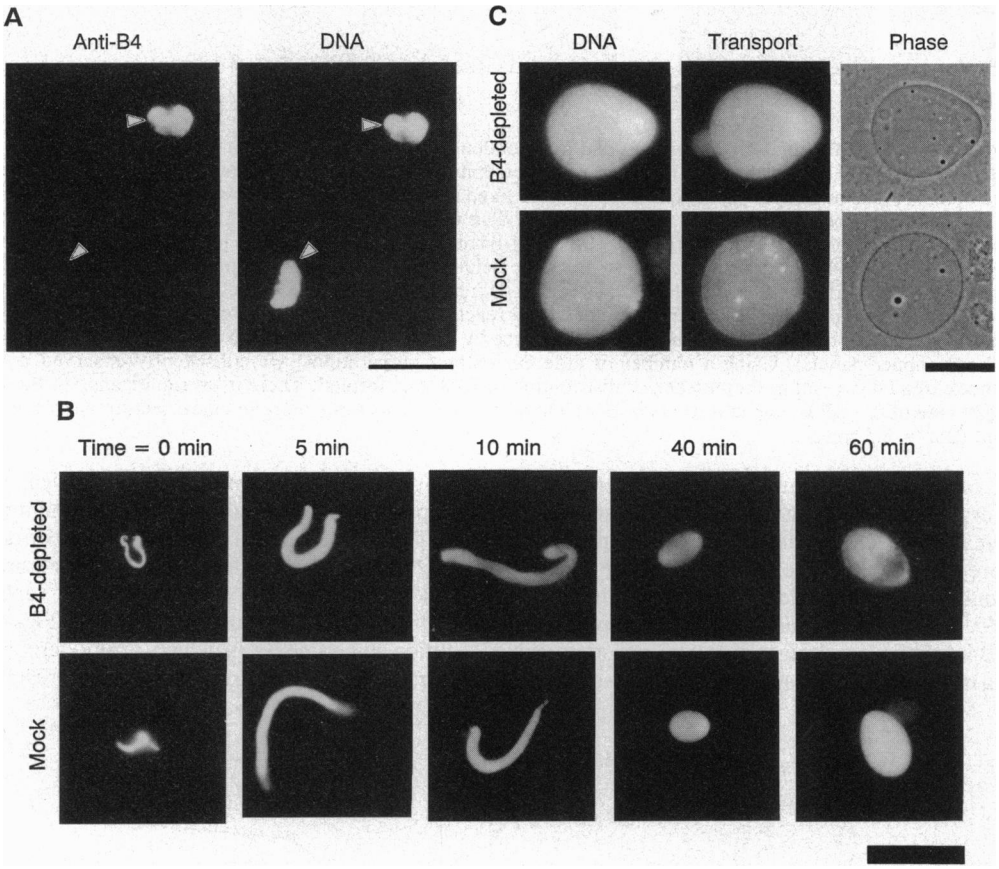

FIG. 2. Immunodepletion of the B4 protein does not alter nuclear formation. (*A*) Nuclei formed in depleted extracts lack the B4 protein. Sperm chromatin was allowed to form nuclei in extracts reconstituted with membranes and either B4-depleted or mock-depleted cytosol (2000 nuclei per μl). These nuclei were fixed after assembling for 2 h at room temperature. The fixation reaction was stopped and the samples were mixed before the nuclei were centrifuged onto coverslips. The nuclei were stained with the DNA dye Hoechst 33258 and processed for indirect immunofluorescence with affinity-purified anti-B4 antibodies. The right panel shows two nuclei (arrowheads) visualized by Hoechst 33258, while the left panel shows the same field visualized by indirect immunofluorescence with anti-B4 antibodies and a rhodamine-labeled mouse anti-rabbit secondary antibody. The upper right nucleus in the left panel stains at a level characteristic of nuclei formed in control extracts, whereas the lower left nucleus in the same panel stains at the level characteristic of nuclei formed in B4-depleted extracts. (Bar = 6 μm.) (*B*) Nuclei form at the same rate in mock- and B4-depleted extracts. Sperm nuclei (Time = 0 min) added to mock-depleted (Mock) or B4-depleted extracts underwent the initial stages of decondensation rapidly in both reconstituted extracts. The nuclei were fully swollen within 5 min in both reactions. Both samples formed complete nuclear envelopes between 20 and 40 min, and the nuclear volumes increased at comparable rates in both reactions (60 min; see also *C*). Nuclei shown are stained with Hoechst 33258. (Bar = 10 μm.) In this experiment the samples were kept separate so that the origin of the nuclei could be confirmed. (*C*) Nuclei formed normally in the absence of B4 protein. Nuclei were formed from sperm chromatin (2000 nuclei per μl) under standard conditions in extracts reconstituted with membranes and either B4-depleted or control (Mock) cytosol. A fluorescently labeled transport substrate consisting of a rhodamine-labeled simian virus 40 large tumor antigen nuclear localization signal peptide coupled to human serum albumin was added to nuclei 2 h after the start of the assembly reaction. Three hours after the addition of substrate, the level of nuclear transport was observed by fluorescent microscopy (Transport). Nuclear assembly was also monitored visually both by fluorescence microscopy with the DNA dye Hoechst 33258 (DNA) and by phase-contrast microscopy (Phase). (Bar = 6 μm.)

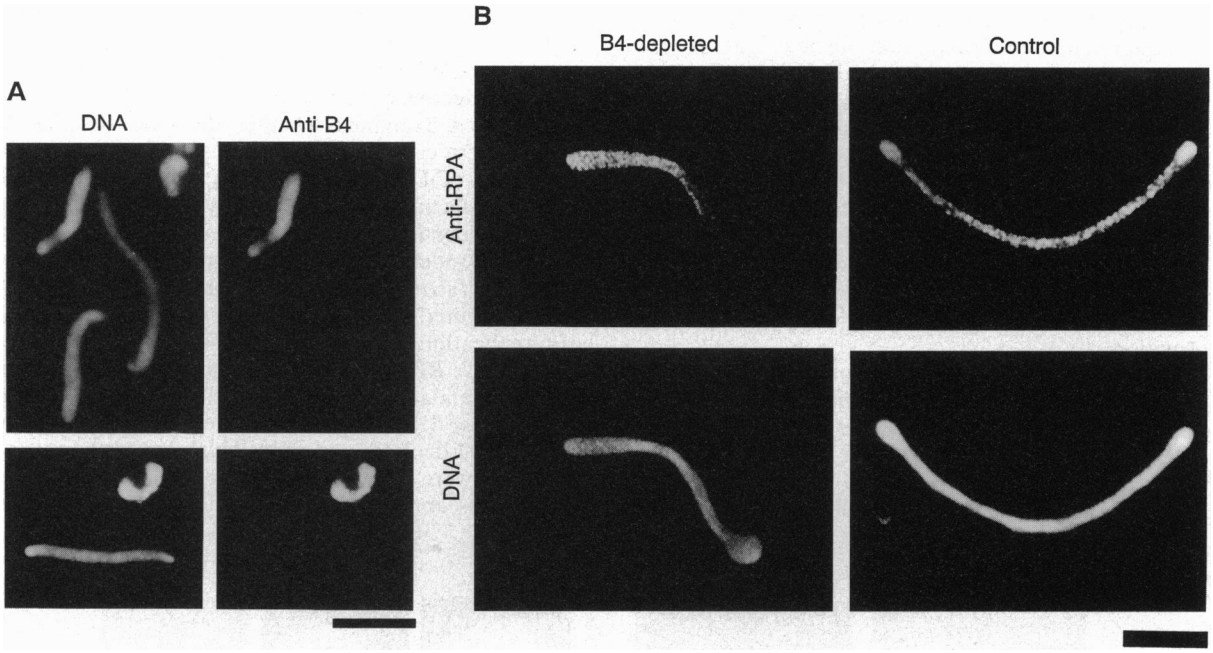

FIG. 3. Immunodepletion of B4 does not alter chromatin decondensation or rearrangement into prereplicative structures. (*A*) Sperm chromatin decondensed in depleted extracts lacks the B4 protein. Demembranated sperm chromatin was allowed to decondense in B4-depleted and mock-depleted cytosol (4000 nuclei per μl). These samples were fixed after 1 h at room temperature. The fixation reaction was stopped and the samples were mixed before being centrifuged onto coverslips. The nuclei were stained with the DNA dye Hoechst 33258 (DNA) and processed for indirect immunofluorescence with affinity-purified anti-B4 antibodies (Anti-B4). The left two panels show representative samples visualized by Hoechst 33258, and the right two panels show the same fields visualized by indirect immunofluorescence with anti-B4 antibodies. (Bar = 5 μm.) (*B*) Demembranated sperm chromatin was allowed to decondense in B4-depleted (left panels) or mock-treated (Control) cytosol (right panels). The decondensed sperm chromatin from each of these reactions was fixed and then centrifuged onto coverslips for staining with Hoechst 33258 (DNA; lower panels) and indirect immunofluorescence by using anti-replication protein A (Anti-RPA) polyclonal rabbit serum as a primary antibody (upper panels). Using a number of different extract preparations, we consistently observed no difference between B4-depleted and mock-treated cytosol in the number or distribution of RPA foci formed. There is no significance to the lack of immunofluorescence on the right side of the RPA-stained nucleus in B4-depleted extracts. This same morphological feature was seen for both control and B4-depleted nuclei. (Bar = 2.5 μm.)

the remodeling of sperm chromatin and the assembly of prereplication centers (30–32). Staining with antibodies against RPA reveals the identical assembly of prereplication centers in the presence or absence of histone B4 (Fig. 3*B*). Each brightly immunofluorescent dot represents a prereplication center (29). The number and density of prereplication centers within B4-deficient and control nuclei are identical (Fig. 3*B* and data not shown). This suggests that the assembly of prereplication centers on the nuclear skeleton does not depend on chromatin structures containing linker histones.

The prereplication centers were converted to replication factories following the addition of membrane fractions (30–32). In depleted and control extracts, replication of DNA is initiated approximately equivalently (Fig. 4). Density substitution experiments in which bromodeoxyuridine was incorporated into replicating DNA that was resolved by equilibrium

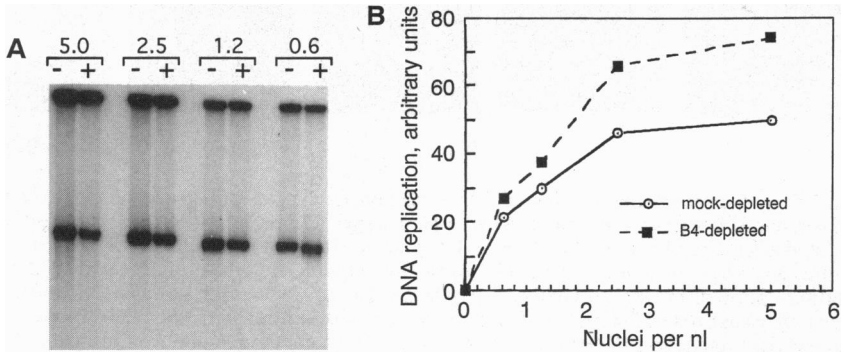

FIG. 4. Immunodepletion of B4 does not prevent DNA replication. (*A*) DNA replication was assayed by the incorporation of [^{32}P]dCTP into high molecular weight DNA. This was done at a series of nuclear concentrations in extracts reconstituted with either B4-depleted (−) or mock-treated (+) cytosol. After 4 h, the reactions were terminated by the addition of sample buffer, and treated as described in *Materials and Methods*. The labeled replication products were then electrophoresed through a 0.8% agarose gel. The concentration of nuclei (nuclei per nl) is indicated above each pair of lanes. (*B*) The ^{32}P-labeled bands in *A* were quantified by using a PhosphorImager (Molecular Dynamics). The amount of DNA replication is shown as a function of the nuclear concentration in B4-depleted (■) and mock-treated (○) samples. We found some variation in the capacity of treated extracts to promote DNA replication; in this case, the B4-depleted extract showed slightly higher incorporation at each nuclear concentration. This observation was consistent between experiments for each extract but was not consistent between extracts, since in other cases the mock-treated extracts had a marginally higher level of nucleotide incorporation. We therefore believe that this slight difference is due to the treatment of the extract rather than to the presence or absence of the B4 protein.

density gradient centrifugation confirmed that semiconservative DNA replication occurs under these conditions (not shown, but see refs. 36 and 37). Thus, functional replication factories, nuclear lamina, and nuclear membrane can be reconstituted within nuclei that do not contain linker histones.

Our results suggest that functional nuclear assembly does not depend upon the presence of intermediate chromatin structures that contain linker histones. This implies that the recognition of chromatin by the nuclear lamina or nuclear membrane depends upon structural features generated by the organization of DNA by the core histones alone or upon specific nonhistone proteins within chromatin. The core histones can direct DNA compaction comparable to that within the chromatin fiber at physiological ionic strengths (38, 39). If the assembly of chromatin loops depends upon this compaction, then the core histones can provide it within *Xenopus* extracts. Alternatively, the assembly of higher-order chromosome structure does not in fact require linker histones or any chromatin structure whose formation they direct (10). The assembly of functional replication factories serves as a control for correct assembly of both the nuclear lamina and the nuclear membrane (30–32). It also demonstrates the lack of an essential role for linker histone in the assembly of a functional nuclear scaffold or in constraining DNA into the looped structures necessary for efficient replication (40).

The *Xenopus* egg extract is a specialized system. For example, it possesses variants of two of the histones: histone H2A.X, which differs from the H2A found in normal somatic cells, and histone B4, a linker histone which differs from the histone H1 found in normal somatic cells. Nuclei assembled in this system are also enriched in the nonhistone protein HMG2 (20). Nevertheless, the failure to find any essential role for linker histones in the assembly of nuclei in this system may explain why certain organisms, such as *Saccharomyces cerevisiae*, can apparently dispense with linker histones (41) and why such a large evolutionary diversity of linker histone structure exists (42, 43). Since linker histones are not essential for DNA compaction into nuclear structures or replication of nuclei, variation in their abundance or structure may serve to modulate the stability of compaction or the efficiency of replication. The *Xenopus* pronuclear reconstitution system provides a direct way to address this idea.

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