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# HIRA Is Critical for a Nucleosome Assembly Pathway Independent of DNA Synthesis

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## Summary

**The mammalian HIRA gene encodes a histone-interacting protein whose homolog in *Xenopus laevis* is characterized here. In vitro, recombinant *Xenopus* HIRA bound purified core histones and promoted their deposition onto plasmid DNA. The *Xenopus* HIRA protein, tightly associated with nuclear structures in somatic cells, was found in a soluble maternal pool in early embryos. *Xenopus* egg extracts, known for their chromatin assembly efficiency, were specifically immunodepleted for HIRA. These depleted extracts were severely impaired in their ability to assemble nucleosomes on nonreplicated DNA, although nucleosome formation associated with DNA synthesis remained efficient. Furthermore, this defect was largely corrected by reintroduction of HIRA along with $(H3\text{-}H4)_2$ tetramers. We thus delineate a nucleosome assembly pathway that depends on HIRA.**

## Introduction

Chromatin assembly is essential to maintain the functional organization of the eukaryotic genome (Verreault, 2000; Mello and Almouzni, 2001). The integrity of this organization can be compromised not only during DNA replication and repair but also during any event that requires chromatin rearrangements (Wolffe and Hansen, 2001), during the cell cycle, or when cell differentiation calls for switches in gene expression programs. At the level of the nucleosome, the fundamental chromatin unit, key factors have been identified that assist the deposition of histones onto DNA to form a core particle (Krude, 1995; Adams and Kamakaka, 1999; Kaufman and Almouzni, 2000). One of these, chromatin assembly factor 1 (CAF-1), is a prime candidate for coupling chromatin assembly to DNA synthesis (Ridgway and Almouzni, 2000). Other chromatin assembly processes take place independently of DNA synthesis, and factors other than CAF-1 are probably involved (e.g., to maintain transcriptionally repressed chromatin states in specific regions of the genome). A predicted prerequisite for such factors is the ability to interact with histones, and candidates can be found in the growing list of histone chaperones (Ito et al., 1997; Philpott et al., 2000). Most such factors have proven efficient in stimulating histone deposition onto DNA in vitro. Considering that in vivo they function in a much more complex environment, it is important to explore whether each factor can be assigned a specific role or if they act redundantly. To address these issues, crude in vitro systems efficient for chromatin assembly can be used to mimic the cellular environment (Kaufman and Almouzni, 2000). *Xenopus* egg extracts that supply various histone chaperones are unique in that they can reproduce synthetic nuclei, initiation of replication, and cell cycle events (Almouzni and Wolffe, 1993). In these experimental systems, the role of each histone chaperone can be evaluated by immunodepletion/complementation experiments that examine its critical importance in a more complex environment and in connection with other aspects of nuclear metabolism. In the present study, we focused our attention on HIRA (Lamour et al., 1995), a protein that was recently found to interact with histones in mammals (Lorain et al., 1998; Magnaghi et al., 1998).

HIRA protein is encoded by a gene within a region of human chromosome 22q11.2 deleted in most patients with the DiGeorge syndrome, a developmental disorder, and was named for its amino acid sequence homology to two *S. cerevisiae* proteins, Hir1p and Hir2p (Lamour et al., 1995; Lorain et al., 1996). Throughout most of the cell cycle, Hir1p and Hir2p tightly repress transcription at the *HTA1-HTB1* locus, which harbors one copy each of the H2A and H2B histone genes (Sherwood et al., 1993; Spector et al., 1997). At the G1/S transition, they transiently recruit a SWI/SNF chromatin remodeling complex, thereby leading to transcriptional activation at this locus (Dimova et al., 1999). Interestingly, deletion of the *HIR* genes and of *CAC2*, whose product is the homolog in *S. cerevisiae* of the p60 subunit of human CAF-1, leads to a synergistic defect in silencing at both telomeres and the silent mating type loci (Kaufman et al., 1998). This epistatic relationship in yeast provided the first functional hint that HIRA and CAF-1 proteins could play overlapping yet distinct roles in chromatin dynamics. This was consistent with the finding that both human and murine HIRA proteins had histone binding properties (Lorain et al., 1998; Magnaghi et al., 1998). Very recently, HIRA has been reported to be essential for normal embryogenesis, but the complex phenotypes of the HIRA knockout mice emphasize how challenging it has been to elucidate the functional relevance of the molecular data accumulated so far (Roberts et al., 2002).

Here, we have examined how HIRA could participate in nucleosome formation in a complex system. We first isolated the *Xenopus laevis* HIRA homolog. In vitro, recombinant *Xenopus* HIRA protein interacted with purified core histones and facilitated their deposition onto

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DNA. In somatic cells, the *Xenopus* HIRA protein was tightly associated with nuclear structures. In embryonic cells, an abundant maternal pool was present whose zygotic replacement was found to ensure constant supplies. *Xenopus* eggs thus appeared as a convenient biochemical source of HIRA protein. Remarkably, a high-speed egg extract (HSE) that had been specifically immunodepleted of HIRA had concomitantly lost its ability to assemble nucleosomes independently of DNA synthesis. In contrast, the same depleted extract remained potent for nucleosome assembly when coupled to nucleotide excision repair (NER) or complementary DNA strand synthesis. Because this defect could be corrected by addition of either HIRA-enriched fractions or a purified recombinant HIRA protein together with $(H3\text{-}H4)_2$ tetramers, we conclude that HIRA plays a critical role in a specific chromatin assembly process that is not coupled to DNA synthesis.

## Results

### *Xenopus* HIRA Interacts with Core Histones and Facilitates Their Deposition onto DNA

*Xenopus* HIRA cDNAs were isolated, and the *Xenopus* HIRA protein sequence was deduced from a full-length cDNA clone. The *Xenopus* HIRA protein is 1013 amino acids long as compared to 1017 amino acids for human HIRA (Figure 1A). The amino acid identity between the two species is 77% overall and greater that 92% between the amino-terminal third (aa 1–355) of the two proteins, which define seven WD repeats that are predicted to assemble into a β propeller structure (Neer and Smith, 2000). This region is also 27% identical to the corresponding region in the p60 subunit of human CAF-1.

We then examined the capacity of the *Xenopus* HIRA protein to interact with core histones. By Far Western blotting, GST-HIRA could bind to all four core histones derived from *Xenopus* A6 cells, calf thymus, or chicken erythrocytes, with H3 and H4 giving the strongest and weakest signals, respectively, and did not bind to BSA (Figure 1B). GST alone did not elicit any signal under similar reaction conditions (data not shown). Thus, the histone-HIRA interactions appeared specific. Moreover, each of the four in vitro translated $^{35}$S-labeled core histones was pulled down with GST-HIRA and not with GST alone (Figure 1C). Using in vitro-translated $^{35}$S-labeled fragments of core histones lacking the amino-terminal tails, we found that only globular (i.e., tailless) H3 was efficiently retained by GST-HIRA versus GST alone (Figure 1C). Notably, detectable amounts of H3 were pulled out from crude extracts prepared from *Xenopus* eggs using an excess of GST-HIRA (Figure 1D).

We next investigated whether HIRA was able to facilitate nucleosome formation in vitro. Purified histones ($[H3\text{-}H4]_2$ tetramers and H2A-H2B dimers) were used at concentrations that alone could not promote efficient histone deposition onto DNA (Earnshaw et al., 1980). Core particle formation was revealed by a faster electrophoretic migration of the initially relaxed DNA plasmid using a classical supercoiling assay (Germond et al., 1979). As shown by the appearance of supercoiled DNA, an untagged recombinant HIRA protein, r-HIRA, but not the r-HIRA elution buffer could promote histone deposition onto plasmid DNA under the same conditions (Figure 1E). As a reference, we used nucleoplasmin, a histone chaperone known to efficiently load histones onto DNA (Figure 1E, lane 16) (Laskey et al., 1978). These results demonstrate that HIRA can promote histone deposition onto DNA in vitro.

### Expression of the *Xenopus* HIRA Protein in Somatic Cells and during Development

Our polyclonal antibody showed the HIRA protein present in nuclear but not cytosolic extracts from *Xenopus* A6 cells, with a staining pattern identical to that obtained using a previously described monoclonal antibody (Figure 2A). It was species specific since it did not produce any bands in the human or murine cell extracts. Proliferating cell nuclear antigen (PCNA), located in both cytoplasm and nucleus (Bravo and Macdonald-Bravo, 1987), was used as a reference (Figure 2A). The subcellular localization of HIRA in somatic A6 cells was confirmed by immunofluorescence studies (Figure 2B). A strong HIRA-specific nuclear staining with a granular pattern was observed in interphase, while in mitosis, staining became dispersed throughout the cell and was excluded from DAPI-stained regions (Figure 2B). The nuclear staining in interphase resisted cell permeabilization with Triton prior to fixation, even when performed after extensive DNA or RNA degradation (Figure 2B). This indicates that HIRA staining in interphasic cells depends on the existence of a stable nuclear component that resists the action of nucleases. For a more quantitative assessment, cells were processed for Western blot analysis. We observed no detectable decrease of HIRA protein staining after Triton treatment as compared with the histones, confirming HIRA resistance to Triton permeabilization (Figure 2B). In contrast, most of the p150 CAF-1 was sensitive to Triton extraction, as reported in human cells (Martini et al., 1998).

Remarkably, HIRA protein was abundant in a soluble form in *Xenopus* high-speed egg extract (HSE) (Figure 2A). During oogenesis, the HIRA protein was present from stage IV, steadily increasing until stage VI without significant variation noted during oocyte maturation (Figure 2C). We estimate that ~5–10 ng of HIRA protein is present per egg. Throughout early embryonic development, HIRA expression remained constant (Figure 2C). PCNA, whose protein levels increase steadily during oogenesis and reach stable levels during embryonic development (Leibovici et al., 1992), was examined on the same blots for comparison purposes (Figure 2C). Thus, the large maternal pool of HIRA provided in early embryonic cells is progressively diluted during cell division, after which a zygotic supply is produced to ensure a stable steady state at later developmental stages.

### HIRA Depletion Affects the Chromatin Assembly Capacity of HSE

We used *Xenopus* high-speed egg extracts (HSE), which are highly competent for chromatin assembly (Laskey et al., 1977; Glikin et al., 1984; Almouzni and Mechali, 1988) and, as seen above, provide a convenient biochemical source of HIRA. The HIRA antiserum was used to immunodeplete HIRA from HSE, resulting in an extract

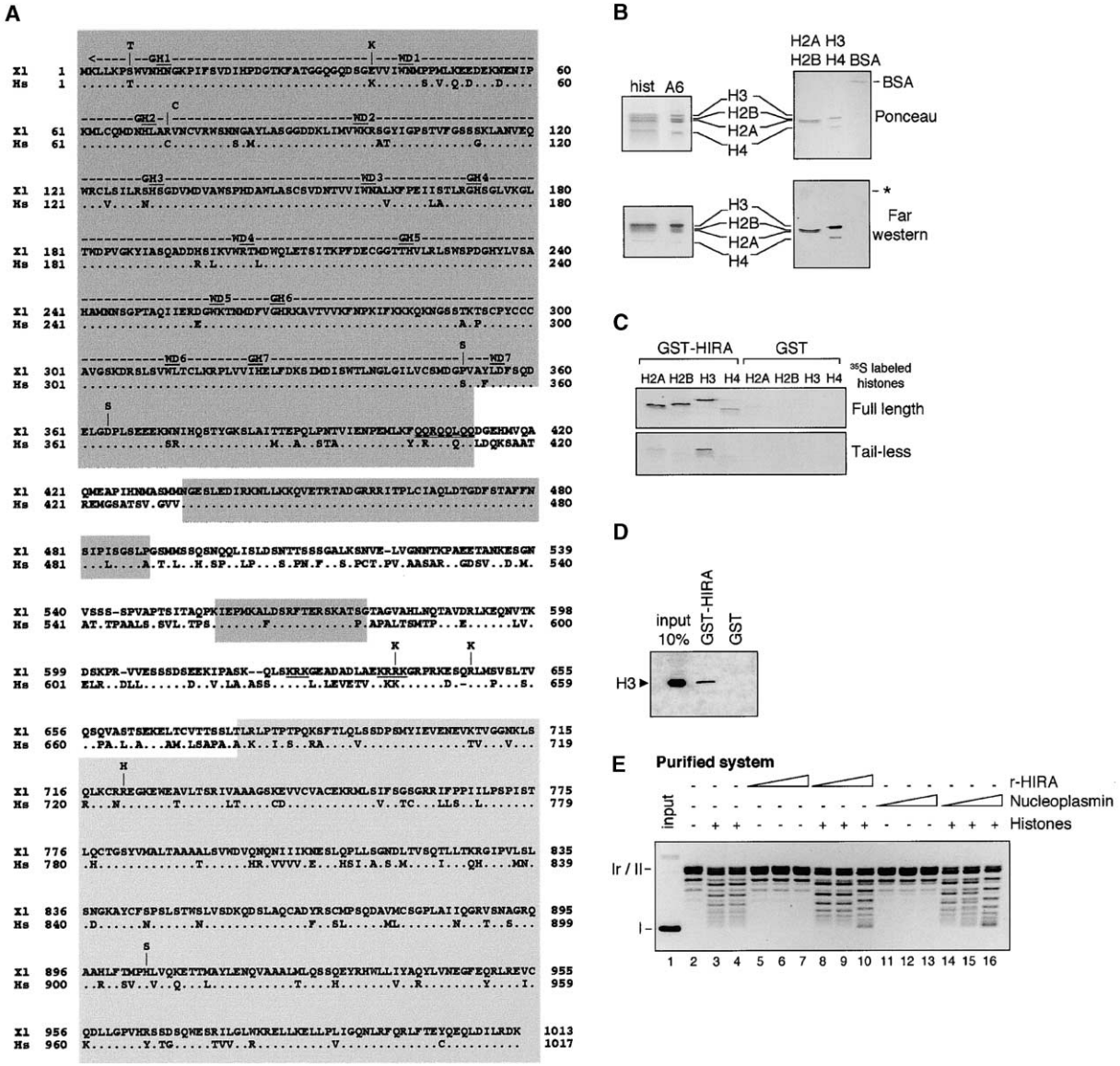


Figure 1. Characterized *Xenopus* HIRA Interacts with the Core Histones In Vitro and Stimulates Reconstitution of Nucleosomal Core Particles onto Plasmid DNA Using Pure Histones

(A) Comparison of the *Xenopus laevis* (Xl) and *Homo sapiens* (Hs, accession number P54198) HIRA protein sequences using the Blast 2 program. Alternative amino acids resulting from nucleotide differences in various *Xenopus* cDNA inserts are displayed above the *Xenopus* (upper) sequence. Identical amino acids are shown as dots in the human (lower) sequence. Dashes in the sequence indicate gaps inserted for alignment purposes. Regions with at least 92% or 77% amino acid identity are shown with a dark or pale shaded background, respectively. The dashed line above the *Xenopus* sequence delineates the WD repeat domain with the characteristic dipeptides marked GH1 to GH7 and WD1 to WD7 (see http://bmerc-www.bu.edu/wdrepeat/welcome.html). A glutamine-rich sequence is doubly underlined. A basic bipartite nuclear localization signal (aa 623–625 and 635–643) is underlined.

(B) 2 μg H2A-H2B dimers and 2 μg $(H3\text{-}H4)_2$ tetramers from chicken erythrocytes, 2.5 μg histones from calf thymus (hist) and *Xenopus* A6 cells, and 1 μg BSA were separated by electrophoresis, transferred to a membrane stained with Ponceau, incubated with GST-HIRA, and then revealed using an anti-GST antibody (Far Western). The asterisk indicates the position of the BSA.

(C) $^{35}$S-labeled full-length and tailless core histones were tested for binding to GST and GST-HIRA.

(D) GST or GST-HIRA (5 μg) was incubated with 10 μl of HSE, and the bound proteins were analyzed by Western blotting using anti-H3 antibodies.

(E) For reconstitution of nucleosomal core particles onto DNA, r-HIRA (~5, 10, and 25 ng), nucleoplasmin (50, 150, or 250 ng), or control r-HIRA elution buffer (lane 4) was combined with topoisomerase I and relaxed plasmid in the presence or absence of histones (H2A-H2B dimers [150 ng] and $[H3\text{-}H4]_2$ tetramers [150 ng]). The purified plasmid products analyzed by agarose gel electrophoresis were visualized by ethidium bromide staining. Input DNA run in parallel (lane 1) and migration positions of DNA plasmid form I (supercoiled), form II (nicked circular), and form Ir (closed circular) are indicated.

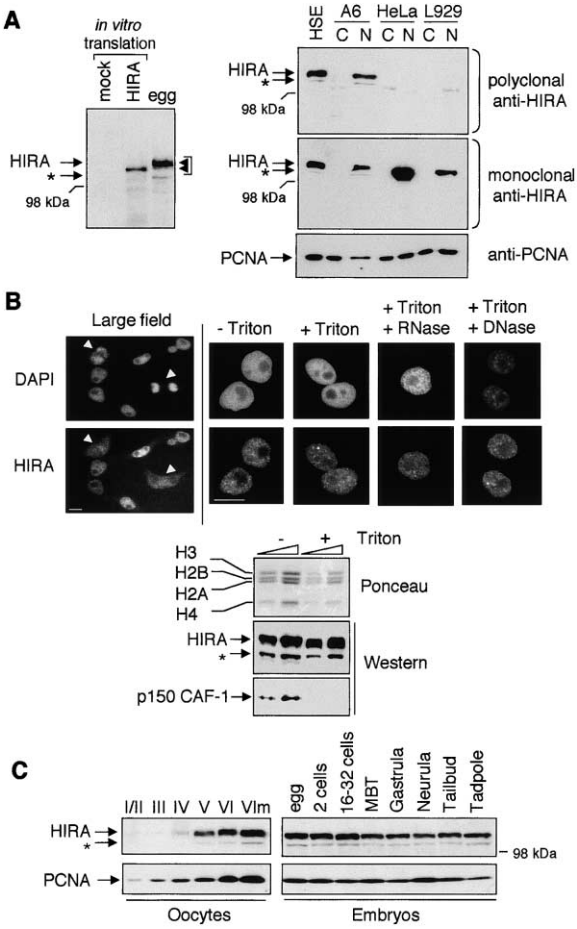


Figure 2. HIRA Expression in Somatic Cells and during *Xenopus* Development

(A) Left: specificity of the HIRA antibody. 1 μl of mock or HIRA in vitro-translated products and material extracted from one unfertilized egg were processed for Western blot analysis and detection with the polyclonal HIRA antibody. The bracket with the arrows indicates a migration shift, which was eliminated by a lambda phosphatase treatment (data not shown). The asterisks in panels (A), (B), and (C) indicate a minor band detected in *Xenopus* extracts by both monoclonal and polyclonal HIRA antibodies, even when affinity purified (data not shown). Right: subcellular HIRA protein expression. Western blotting was performed using 20 μg of HSE, nuclear (N) or cytosolic (C) extracts from *Xenopus* A6, human HeLa or murine L929 cells, and either the polyclonal or the monoclonal HIRA antibody. As control, a PCNA antibody was used on the same membrane. Migration position of the 98 kDa molecular weight standard is indicated.

(B) The HIRA immunolocalization is Triton, RNase, and DNase resistant in *Xenopus* A6 cells. HIRA was localized using the affinity-purified polyclonal HIRA antibody, and DNA was visualized by DAPI counterstaining. A6 cells were either directly fixed or extracted with Triton before fixation in the absence or in the presence of 0.2 mg/ml DNase I or 0.1 mg/ml RNase A. Arrows show mitotic cells. Scale bars are 5 μm. A quantitative assessment was performed by Western blot analysis of cellular extracts from A6 cells treated (Triton) or not treated (−) with Triton and using polyclonal HIRA and p150 CAF-1 antibodies. As a loading control, histones were stained with Ponceau. Cellular extracts corresponding to $2 \times 10^5$ and $4 \times 10^5$ cells show that signal detection was in a linear range.

(C) *Xenopus* HIRA expression during oogenesis and embryonic development. Protein extracts (equivalent to one oocyte or embryo) (Verheggen et al., 1998) were subjected to Western blot analysis as in ([A], left).

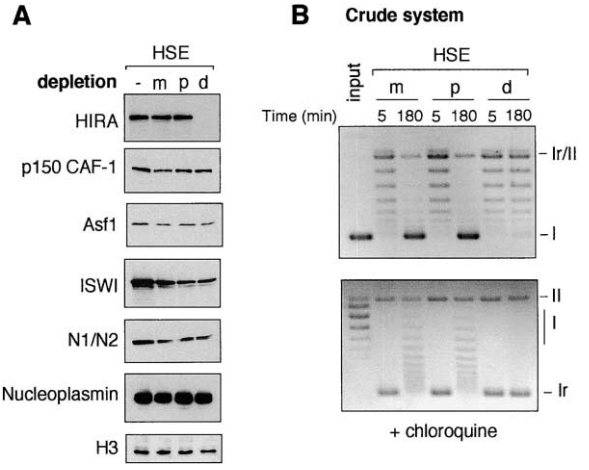


Figure 3. Depletion of HIRA Reduces the Nucleosome Assembly Activity of HSE

(A) Control HSE (−) and mock (m), preimmune (p), and HIRA-depleted (d) HSE were analyzed by Western blotting using the indicated antibodies.

(B) Plasmid supercoiling analysis was carried out after the indicated incubation times (5 or 180 min) by analysis on agarose gel in the absence or the presence of chloroquine (10 μg/ml). The migration positions of plasmid are indicated as in Figure 1E.

that was >90% depleted of HIRA protein (HSEd) (Figure 3A). That the depletion was specific for HIRA was demonstrated by the observation that the levels of other *Xenopus* histone interacting proteins, including the p150 subunit of CAF-1 (Quivy et al., 2001), Asf1 (Sillje and Nigg, 2001), N1/N2, nucleoplasmin (Dilworth et al., 1987), and the ISWI subunit present in several chromatin remodelling complexes (Guschin et al., 2000), had remained unchanged during the procedure (Figure 3A). Excess of exogenous HIRA was necessary to pull down histone H3 from *Xenopus* egg extract in vitro (Figure 1D). However, amounts of H3 were virtually constant in the extract prior to and following HIRA immunodepletion (Figure 3A) and below detection level in the HIRA immunoprecipitate (data not shown). Thus, only a small proportion of the total H3 pool is potentially engaged in this interaction.

The nucleosome assembly activity present in this HIRA-depleted extract was examined using the plasmid supercoiling assay (Figure 3B). After a 5 min incubation in the various extracts, the initially supercoiled plasmid was mainly in the relaxed form (form Ir), reflecting the presence of active topoisomerase in all extracts. After 3 hr in the presence of the control HSEm or HSEp extracts, the rapidly migrating form I had reappeared, demonstrating the presence of nucleosome assembly activity in these extracts. In contrast, very little form I reappeared when incubation took place in HIRA-depleted extract (HSEd) even after longer incubation times, indicating that the component(s) responsible for the supercoiling activity had been removed during the HIRA depletion procedure. In order to ensure that the lack of supercoiling was not due to the accumulation of nicked DNA that would migrate along with relaxed plasmids, we used chloroquine-containing gels. In the presence of HIRA-depleted extracts, the plasmids were

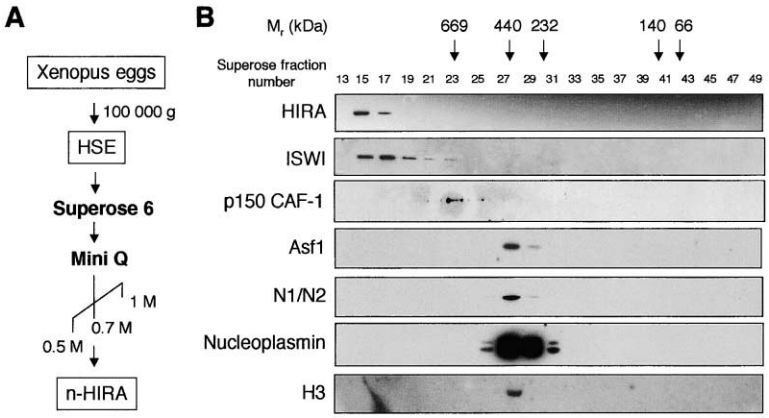


Figure 4. Preparation of the Native HIRA Fraction, n-HIRA, from HSE

(A) Preparation scheme.

(B) The Superose 6 column fractions were analyzed by Western blotting using the indicated antibodies. Molecular weight standards are indicated.

indeed predominantly in relaxed form after a 3 hr incubation (Figure 3B). We conclude that HIRA depletion has a profound effect on the capacity of *Xenopus* egg extracts to assemble nucleosomes. This effect is remarkable considering the amount of other putative assembly factors still present in the depleted extracts.

### Addition of Both HIRA and $(H3\text{-}H4)_2$ Tetramers Is Required to Restore a Significant Chromatin Assembly Capacity in HIRA-Depleted HSE

We next tested whether the readdition of HIRA protein to the depleted HSE could reconstitute its activity using two different sources of HIRA proteins, one recombinant (r-HIRA) and the other native (n-HIRA), obtained as described in Figure 4A. After the Superose 6 column, HIRA eluted within high molecular mass fractions (above 670 kDa), indicating that HIRA is present in a large complex in the HSE (Figure 4B). These fractions were analyzed using antibodies recognizing histone H3 and proteins involved in nucleosome assembly (nucleoplasmin, N1/N2, p150 CAF-1, Asf1) or chromatin remodelling (ISWI). Remarkably, none of these proteins cofractionated with HIRA at this stage of purification except ISWI (Figure 4B). These fractions were further loaded on a miniQ column to obtain native n-HIRA. This preparation, which also contained ISWI (data not shown), was used in our assays.

We first verified that adding small amounts of extracts to the HIRA-depleted HSE (HSEd) did restore the plasmid supercoiling activity in a dose-dependent fashion (Figure 5A, lanes 4 and 5). This excluded the possibility that the supercoiling defect was the result of a contaminating inhibitory activity introduced in the course of the HIRA-specific depletion procedure. We found that purified r-HIRA was not sufficient by itself to restore significant chromatin assembly capacity in the HIRA-depleted extract (Figure 5A, lane 8). Since HIRA interacts with core histones in vitro, we tested whether the addition of a combination of r-HIRA and histones could complement the depleted extract. When $(H3\text{-}H4)_2$ tetramers, but not H2A-H2B dimers, were added together with r-HIRA, we observed a significant improvement in supercoiling activity of the depleted extract (Figure 5A, lane 10). In contrast, neither $(H3\text{-}H4)_2$ tetramers nor H2A-H2B dimers alone exhibited the same complementing capacity (Figure 5A, lanes 7 and 6). No further improvement of supercoiling activity was observed in the extract when H2A-H2B dimers were added together with $(H3\text{-}H4)_2$ tetramers (data not shown). The native n-HIRA was also functional together with $(H3\text{-}H4)_2$ tetramers to complement the assembly defect of HSEd (Figure 5A, lane 14).

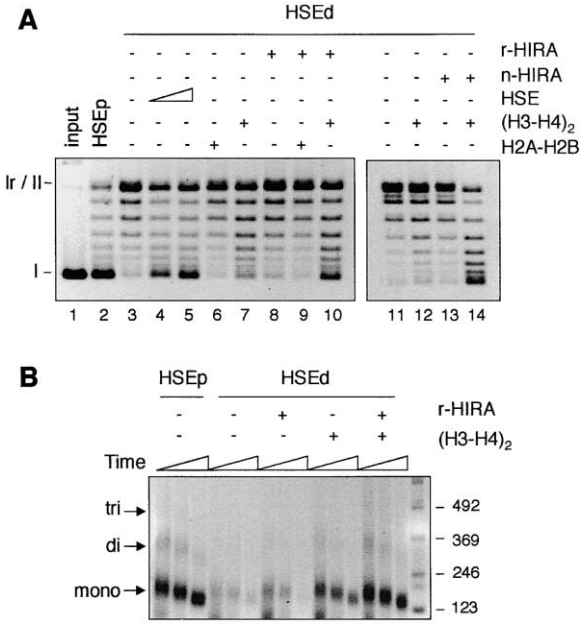


Figure 5. Addition of Both HIRA and $(H3\text{-}H4)_2$ Tetramers Significantly Restores the Nucleosome Assembly Activity of HIRA-Depleted HSE

(A) Limited amounts of HSE (1 or 2 μl) or various combinations of r-HIRA (~5 ng) or n-HIRA (4 μl) and core histones (150 ng of H2A-H2B dimers or 150 ng of $[H3\text{-}H4]_2$ tetramers) were added to the reaction mix in HIRA-depleted HSE (HSEd). DNA products were analyzed after 3 hr incubation in parallel with input DNA by agarose gel electrophoresis. The migration positions of plasmid are indicated as in Figure 1E.

(B) Nucleosome assembly reactions performed with r-HIRA (about 5 ng) and/or 150 ng of $(H3\text{-}H4)_2$ tetramers in the presence of HIRA-depleted HSEd or control HSEp were subjected to extensive micrococcal nuclease (MNase) digestion for increasing incubation times (0.5, 1, and 3 min). The resulting DNA fragments were analyzed by agarose gel electrophoresis. Positions of mononucleosome (mono), dinucleosome (di), and trinucleosome (tri) are indicated. The 123 bp ladder (GIBCO-BRL) is indicated in base pairs.

In this case, the rescue appeared even more efficient than with the recombinant protein, despite the fact that the amount of HIRA protein added was at least 10-fold less, as estimated by Western blotting (data not shown).

In parallel experiments, the various assembly products were analyzed by extensive microccocal nuclease (MNase) digestion in order to estimate the proportion of protected fragments corresponding to nucleosome size. Here, for similar input DNA, in the presence of preimmune-depleted HSE (HSEp), MNase treatment generated protected DNA that migrated at positions corresponding to mono-, di-, and trinucleosomes (Figure 5B). In contrast, little protected DNA was recovered when MNase treament was performed in the presence of HIRA-depleted extract (HSEd) (Figure 5B), consistent with a histone deposition reaction impaired after HIRA depletion. Our complementation experiments revealed that, although a slight increase in the proportion of mononucleosome could be obtained with addition of $(H3\text{-}H4)_2$ alone, a better protection was achieved when both r-HIRA and $(H3\text{-}H4)_2$ tetramers were added together (Figure 5B). Thus, the depletion and complementation experiments together argue for a critical role of HIRA in a nucleosome formation process in the complex context of the *Xenopus* egg extract.

### HIRA Depletion Does Not Affect Chromatin Assembly Activity Coupled to DNA Synthesis

In *Xenopus* egg extracts, chromatin assembly is very efficient when coupled to DNA synthesis in the course of either complementary strand synthesis (Almouzni and Mechali, 1988; Almouzni et al., 1990) or nucleotide excision repair (NER) (Gaillard et al., 1996). We tested whether these specific assembly pathways were altered in HIRA-depleted extracts. To investigate chromatin assembly following NER (Gaillard et al., 1999), nucleosome assembly reactions were performed on UV-irradiated plasmids in the presence of [$\alpha$-$^{32}$P]dCTP and the different HSE, and the topology of both total and repaired DNA was analyzed. As expected, little supercoiling activity was detected when either control or UV-irradiated plasmids were incubated in the HIRA-depleted extracts (HSEd) (Figure 6A, Total). In contrast, analysis of the radiolabeled repaired DNA (Figure 6A, Labeled) showed that supercoiling was not affected in depleted extracts (HSEd). To confirm this result, we followed nucleosome assembly coupled or uncoupled to DNA repair simultaneously in the same reaction using two plasmids of different sizes that could be distinguished by gel migration. Again, we found that the HIRA-depleted extract HSEd failed to assemble the nonirradiated plasmid whereas the repaired UV-treated plasmid was efficiently assembled (Figure 6B). In contrast, the control HSEp generated nucleosomal assembly when coupled or uncoupled to DNA synthesis. Thus, nucleosome assembly activity linked to DNA repair was not significantly altered by HIRA depletion. However, since only a small fraction of the molecules—about 10%–15%—are repaired in this experimental setup (Gaillard et al., 1997), the small percentage of HIRA molecules remaining in the depleted extract may be sufficient to induce the supercoiling observed after irradiation. To exclude this possibility, we used a system in which nucleosome assembly occurs

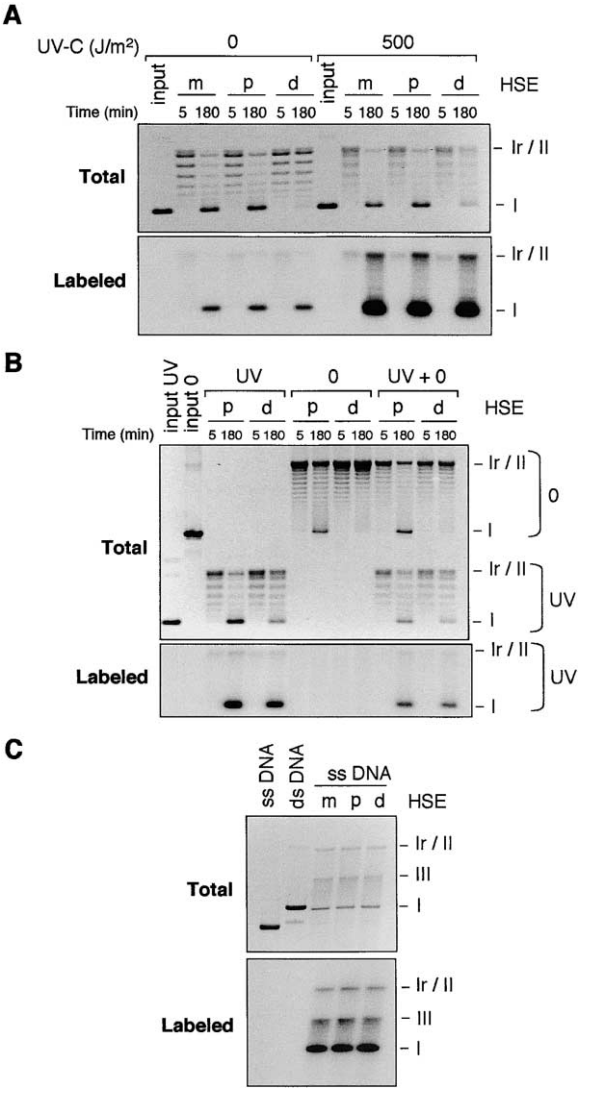


Figure 6. HIRA Depletion Does Not Affect the DNA Synthesis-Coupled Nucleosome Assembly Activity of HSE

(A) The nucleosome assembly activity of HSE coupled to NER is not affected by HIRA depletion. Supercoiling assays were performed in presence of [$\alpha$-$^{32}$P]dCTP using either nontreated plasmid (0) or plasmid irradiated with 500 J/m$^2$ UV-C (500) and mock (m), preimmune (p), or HIRA-depleted (d) HSE. After 5 or 180 min incubation, DNA was analyzed on agarose gel and visualized by ethidium bromide (Total) and by autoradiography (Labeled). The migration positions of plasmid are indicated as in Figure 1E.

(B) The nucleosome assembly activities of HSE coupled and uncoupled to nucleotide excision repair were tested in the same reaction by using two plasmids of different size, one was irradiated at 500 J/m$^2$ UV-C (UV) and the other was not (0). Supercoiling assays were performed as in (A).

(C) The nucleosome assembly activity coupled to complementary DNA strand synthesis is not affected by HIRA depletion from HSE. Reactions were carried out using single-stranded M13 bacteriophage DNA and mock (m), preimmune (p), or HIRA-depleted (d) HSE in the presence of [$\alpha$-$^{32}$P]dCTP. The DNA products were analyzed as in (A). The single-stranded DNA (ss DNA) and double-stranded circular DNA (ds DNA) migrated in parallel. The migration positions of form I (supercoiled), form II (nicked circular), form Ir (closed circular), and form III (linear) are indicated.

in conjunction with complementary strand synthesis. Reactions were carried out with the different extracts using single-stranded M13 DNA as a template in the presence of [$\alpha$-$^{32}$P]dCTP to follow the fate of newly synthesized DNA, and both total and labeled DNA were analyzed. At the end of the reaction, all single-stranded DNA molecules were converted into double-stranded forms in both HSEd and control extracts (HSEm and HSEp) (Figure 6C, Total). In all cases, the resulting labeled DNA was mostly supercoiled (Figure 6C, Labeled). Thus, a HIRA-depleted extract, whose capacity to assemble chromatin on nonreplicating DNA was dramatically decreased, exhibited no detectable defect in nucleosome assembly on newly synthesized DNA.

## Discussion

Studies in *S. cerevisiae* have demonstrated that the yeast homologs of HIRA are involved in transcriptional coregulation at the level of chromatin (Sherwood et al., 1993; Spector et al., 1997; Dimova et al., 1999). However, the precise mechanism of this activity has been unclear. Using a frog family member, we demonstrated that HIRA is critical in a specific chromatin assembly pathway that takes place independently of DNA synthesis. In light of the high degree of sequence conservation between family members (Figure 1A) (Lamour et al., 1995; Scamps et al., 1996; Roberts et al., 1997; Kirov et al., 1998; Llevadot et al., 1998) and since histone interactions appear to occur in all species where they have been tested for (Figure 1) (Lorain et al., 1998; Magnaghi et al., 1998), we predict that this function will be relevant in a wide range of eukaryotic organisms.

That HIRA facilitates the deposition of purified histones onto plasmid DNA in vitro suggested a functional significance for the interaction of HIRA with histones (Figure 1). Depletion experiments revealed a severe reduction in the capacity of *Xenopus* egg extracts depleted for HIRA to promote nucleosome formation in the absence of DNA synthesis (Figures 3, 5, and 6). This defect was not due simply to histone depletion, because we found that the pool of histone H3 was not significantly reduced. Furthermore, no major change was observed in the pool of histone-interacting proteins, including N1/N2, nucleoplasmin, Asf1, or the p150 subunit of CAF-1. Importantly, the addition of both r-HIRA and $(H3\text{-}H4)_2$ tetramers, but not H2A-H2B dimers, to the depleted extracts significantly restored the capacity of the extracts to assemble chromatin independently of DNA synthesis. A more efficient reconstitution was obtained when n-HIRA, prepared from HSE, was added with $(H3\text{-}H4)_2$ tetramers. This suggested either that the recombinant protein lacked some postranslational modifications or that a partner in n-HIRA participated in the complementation, or both. Since HIRA is phosphorylated at various stages of the human cell cycle (De Lucia et al., 2001; Hall et al., 2001) and is also phosphorylated in HSE (data not shown), r-HIRA is possibly not adequately modified. Alternatively, additionnal partners may be required for full function in the extract. The only tested protein that cofractionated with n-HIRA in our procedure was ISWI, the ATPase component of several chromatin remodelling factors. ISWI is a subunit in the human RSF complex (Loyola et al., 2001), which promotes nucleosome formation in a reconstituted system with pure histones. No major depletion of ISWI was observed in our experiments. However, since ISWI is present in several complexes, it remains possible that only the fraction that is part of a putative RSF complex was depleted. Interestingly, the addition of antibodies against *Xenopus* ISWI in egg extracts was reported to affect regular nucleosomal spacing in an assembly reaction without causing major histone loading defects, suggesting that ISWI present in n-HIRA is probably not critical for histone deposition (Guschin et al., 2000; MacCallum et al., 2002). Future biochemical characterization of n-HIRA is required to better understand how HIRA itself plays a critical role in the assembly pathway. The reproducibility of the rescue with the specific combination of HIRA and $(H3\text{-}H4)_2$ tetramers argues that HIRA exists in a transient complex with a critical pool of $(H3\text{-}H4)_2$ that is necessary for an efficient assembly reaction independent of synthesis. It may appear surprising that additional $(H3\text{-}H4)_2$ tetramers are required for the complementation since high levels are already present in the extract. Possibly, the endogenous pool of histones, already engaged in other complexes, is not available for an interaction with exogenous HIRA. Indeed, as a precedent, the importance of phosphorylation has been reported for nucleoplasmin to release histones (Cotten et al., 1986). Thus, the concept of a slow, passive assembly pathway, whereby a simple release of histones from the histone storage pool accounts for the formation of nucleosomes on nonreplicated DNA, seems unlikely. Instead, we propose that HIRA complexed with $(H3\text{-}H4)_2$ tetramers may be necessary to initiate nucleosome formation.

Importantly, our depletion experiments did not affect chromatin assembly linked to DNA synthesis. This pathway is active in the *Xenopus* system (Gaillard et al., 1996; Kamakaka et al., 1996; Quivy et al., 2001) and dependent on chromatin assembly factor CAF-1 and PCNA (Shibahara and Stillman, 1999; Moggs et al., 2000). Our findings thus suggest that HIRA and CAF-1 proteins are not required in the same chromatin assembly pathways and that HIRA plays a major role in nucleosome formation that is not coupled to DNA synthesis. This role is thus different from that proposed for other histone chaperones, such as nucleoplasmin and N1/N2. Indeed, depletion of nucleoplasmin or N1/N2 from *Xenopus* egg extracts affects nucleosome assembly both in the presence or absence of DNA synthesis (Dilworth et al., 1987; Kleinschmidt et al., 1990). Removing these chaperones, the major histone binding proteins in *Xenopus* egg, also affects the pool of stored histones, thereby leading to defects in nucleosome formation due to a lack of substrates. Thus, the emerging picture is that, in *Xenopus* eggs, the pool of stored H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers is mainly associated with nucleoplasmin and N1/N2, respectively, and that these histones are transferred to dedicated chaperones within various nucleosome assembly pathways that ultimately lead to histone deposition onto DNA. In light of our data, it is tempting to speculate that the maternal store of HIRA at early stages of development could act to control the loading of histones, and thus help to optimize the assembly reaction during rapid cellular division. We found previously that CAF-1 was critical during this time

period, arguing for a need during these rapid divisions for the tight coordination of DNA synthesis and nucleosome assembly (Quivy et al., 2001). In this scenario, any region that would have escaped assembly during DNA synthesis would have a second chance to be assembled later using the HIRA-dependent pathway. At later stages in somatic cells, a DNA synthesis-independent pathway would ensure a secured assembly into nucleosomes outside of S phase at dedicated genomic regions. Such a mechanism might be adapted for transcriptional regulation in regions that are actively transcribed during S phase and thus probably escape CAF-1-mediated chromatin assembly. In this hypothesis, the *S. cerevisiae HTA1-HTB1* histone gene locus, which is repressed outside the G1/early S phase by a mechanism that depends on the Hir1/Hir2 proteins (Sherwood et al., 1993; Spector et al., 1997), provides a coherent picture. In addition, HIRA could mediate an assembly pathway partially redundant with CAF-1 for regions that should be silenced at all phases of the cell cycle. This model is consistent with the observation that in *S. cerevisiae*, the phenotype of *CAC* mutants is exacerbated when *HIR* genes are inactivated, leading to a reduction of silencing in telomeric regions and mating type loci (Kaufman et al., 1998). Moreover, cac$\Delta$ hir$\Delta$ double mutants exhibit centromeric alterations implicating both CAC and HIR in the establishment of this specialized chromatin (Sharp et al., 2002). Furthermore, HIR proteins were reported to act in concert with the histone chaperone Asf1 to promote silencing in yeast (Sharp et al., 2001). This latter factor also synergizes with CAF-1 in stimulating nucleosome formation (Tyler et al., 1999; Sharp et al., 2001). Whether a similar network of interactions exists among HIRA, Asf1, and CAF-1 in higher eukaryotes remains to be investigated.

Taken together, our results emphasize the existence of distinct chromatin assembly pathways. The unexpected critical role of HIRA in promoting chromatin assembly that is independent of DNA synthesis in a complex system without acting by titrating histones is to our knowledge unusual. This assembly pathway that is uncoupled to DNA synthesis and dependent on HIRA may not be completely distinct from other assembly pathways. It will now be necessary to explore the network of interactions throughout the entire assembly line from sites of histone synthesis to their delivery. The situation in egg extracts, which reflects embryonic properties, will have to be analyzed in somatic cells. Unravelling the dynamics of these networks during development and the cell cycle will be critical for our understanding of the functional maintenance of genome organization.

## Experimental Procedures

### Cloning of *Xenopus* HIRA cDNAs

We probed a *Xenopus laevis* cDNA library from XTC cells with a 620 bp-long BamHI-KpnI fragment from the 3′ end of the human HIRA cDNA coding region (Lamour et al., 1995), yielding a partial clone X7 (accession number AJ278386), which was used for a second probing and yielded the overlapping clone X26 (accession number AJ278387). The 5′ end of the *Xenopus* HIRA transcript (clone EX1, acccession number AJ278388) was obtained by PCR amplification using DNA from an oocyte cDNA library (Lemaire et al., 1995) next used to generate a cDNA encoding a full-length protein (accession number AJ404369).

### Recombinant *Xenopus* HIRA Proteins and Generation of a Polyclonal HIRA Antibody

PCR fragments encoding either the full-length or amino acids 401–1013 of the *Xenopus* HIRA protein in the pTYB2 vector were used to produce untagged proteins (Impact T7 Purification System; New England Biolabs, Beverly, MA). A rabbit polyclonal anti-HIRA antiserum was obtained by immunization with the partial untagged recombinant *Xenopus* HIRA protein (Agrobio, Villeny, France) and was affinity purified against the *Xenopus* HIRA-intein fusion protein blotted onto nitrocellulose.

### GST-HIRA Fusion Protein, In Vitro Translated Proteins, and Protein Interactions

The GST-HIRA protein was produced using a PCR fragment containing the coding region of the *Xenopus* HIRA cDNA inserted in the pGEX4T1 expression vector. A TNT-coupled rabbit reticulocyte lysate kit (Promega, Madison, WI) was used for in vitro translation of the full-length *Xenopus* HIRA cDNA clone in the pBluescript II KS+ vector or full-length and tailless *Drosophila* core histones clones in PET21 plasmid (Hamiche et al., 2001). GST-fusion proteins (5 $\mu$g) immobilized on Glutathione sepharose beads and either in vitro translated $^{35}$S-labeled proteins or HSE were incubated for 2 hr at 4°C in binding buffer (20 mM HEPES-KOH [pH 7.4], 150 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP40, and protease inhibitors).

### Manipulation of *Xenopus* Oocytes, Eggs, and Embryos

Oocytes were surgically removed from female *X. laevis* (Kay, 1991) and sorted (Dumont, 1972). Maturation of stage VI oocytes was obtained with 2 $\mu$g/ml progesterone (Sigma, St. Louis, MO) (Bellier et al., 1997). Eggs of female *X. laevis* were obtained after hormone stimulation and were fertilized (Kay, 1991) to produce various staged embryos (Nieuwkoop and Faber, 1956).

### Cell Extracts and Immunofluorescence Analysis

Cytosolic extracts from various cells were prepared using a low-salt buffer (Martini et al., 1998). The nuclear pellet was extracted with 1 M NaCl for nuclear extract preparation. *Xenopus* A6 cells (Rafferty, 1969) were either fixed immediately with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS or prior to fixation treated by: (1) Triton extraction with 0.5% Triton X-100; (2) Triton-DNase extraction using 0.2 mg/ml DNase I (Roche Molecular Biochemicals, Mannheim, Germany) with 0.5% Triton X-100; (3) Triton-RNase extraction using 0.1 mg/ml of RNase A (Roche Molecular Biochemicals) with 0.5% Triton X-100. Affinity-purified HIRA antibody at a 1:250 dilution was visualized with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).

### Western and Far Western Blot Analysis

Protein samples in Laemmli buffer were resolved on SDS-PAGE and transferred to nitrocellulose. Specific proteins were visualized using *Xenopus* HIRA antibody at a dilution of 1:10,000, human HIRA monoclonal antibody (WC15 + WC119) (Hall et al., 2001), human PCNA (monoclonal PC10; Dako, Glostrup, Denmark), *Xenopus* ISWI (Guschin et al., 2000), human H3 (C-16; Santa Cruz Biotechnology, Santa Cruz, CA), human Asf1 (Sillje and Nigg, 2001), *Xenopus* p150 CAF-1 (Quivy et al., 2001), and nucleoplasmin and N1/N2 (Dilworth et al., 1987), and were revealed with secondary antibodies (Jackson ImmunoResearch Laboratories) and a Supersignal detection kit (Pierce, Rockford, IL). For Far Western analysis, histones from various sources and BSA, separated on 18% SDS-polyacrylamide gel, were tranferred to nitrocellulose and incubated with 10 ng/ml GST-HIRA or GST at 4°C overnight in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% nonfat dry milk. A polyclonal anti-GST antibody revealed the interaction.

### Preparation of Native HIRA Protein from HSE

n-HIRA was fractionated at 4°C using the Akta purifier system (Amersham Pharmacia Bioscience, Uppsala, Sweden). 100 $\mu$l of HSE was injected on a superose 6 column at 0.5 ml/min in buffer P (10 mM HEPES [pH 7.5], 70 mM KCl, 0.1 mM DTT, protease inhibitors, and 10% glycerol), and fractions containing HIRA were detected by Western blotting. Pooled fractions from three superose 6 columns were loaded on a mini Q 4.6/50 column at 1 ml/min in buffer P,

unbound material was washed with 20 ml of buffer P, and salt elution was performed by applying a gradient from 0.5 M to 1 M NaCl in buffer P. HIRA-containing fractions eluted at 0.7 M NaCl were concentrated on a Microcon YM-30 (Amicon Millipore, Bedford, MA).

**Nucleosome Reconstitution Assay with Histone Chaperone**

Relaxed pBluescript (pBS) plasmid DNA (0.1 μg) and purified r-HIRA protein or purified nucleoplasmin and/or core histones, ($[H3\text{-}H4]_2$ tetramers, and H2A-H2B dimers purified from chicken erythrocytes) (Simon and Felsenfeld, 1979), in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml BSA, including topoisomerase I in a 50 μl final volume were incubated for 1 hr at 37°C.

**HIRA Depletion and Nucleosome Assembly Reactions in HSE**

For immunodepletion, 1 ml of HSE (Almouzni, 1998) was mixed with an equal volume of anti-HIRA antiserum bound to protein A sepharose for 1 hr at 4°C. Depletion was assessed by Western blot analysis prior to use in chromatin assembly reactions. As controls, HSE were treated with noncoupled protein A sepharose or with protein A sepharose coupled to the preimmune serum. For in vitro nucleosome assembly reactions, pBS plasmid (150 ng) either mock or UV-C irradiated (500 J/$m^2$) was incubated with HSE (8 μl) at 23°C in the presence of [α-$^{32}$P]dCTP (5 μCi) (Gaillard et al., 1996, 1999). The two plasmids, pBS UV-C irradiated (500 J/$m^2$) (3.2 kb) and pTYB1 (7.2 kb) in equal molar amounts, were incubated simultaneously with HSE. Complementary strand synthesis was performed using single-stranded M13 DNA (150 ng) in the presence of [α-$^{32}$P]dCTP (2 μCi). The histones or/and HIRA proteins were added in the complementation reaction after 5 min of incubation. Histones and HIRA proteins were preincubated for 10 min on ice before addition. The supercoiling and DNA synthesis were examined by agarose gel electrophoresis in 1× TAE buffer, with or without chloroquine (10 μg/ml), and visualized by staining with ethidium bromide and by autoradiography. For MNase digestion, 750 U/ml of MNase was used at 23°C and then DNA purified and analyzed by agarose gel electrophoresis in 0.5× TBE buffer.

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