The Histone Binding Protein Nucleoplasmin Does Not Facilitate Binding of Transcription Factor IIIA to Nucleosomal *Xenopus laevis* 5S rRNA Genes[†]

LeAnn Howe,[‡] Toru Itoh,[§] Chiaki Katagiri,[§] and Juan Ausio*[,‡]

Department of Biochemistry and Microbiology, University of Victoria, Victoria, B.C., V8W 3P6, Canada, and Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060, Japan

Received November 3, 1997; Revised Manuscript Received December 9, 1997

ABSTRACT: In an attempt to understand the mechanism by which transcription factors compete with histone octamers for cognate binding sites in chromatin, the effect of the histone binding protein nucleoplasmin on the binding of TFIIIA to nucleosomal 5S rRNA genes was tested. In this study, it was shown that, despite the previously reported nucleosome remodeling ability of nucleoplasmin, the binding of TFIIIA to nucleosomal DNA cannot be facilitated by this protein. Furthermore, it was demonstrated that nucleoplasmin cannot overcome nucleosome mediated repression of transcription of reconstituted 5S rRNA genes. In contrast to earlier work, this study used a homologous system composed of the 5S rRNA gene, nucleoplasmin, and TFIIIA from *Xenopus laevis*.

The phosphoprotein nucleoplasmin is the most abundant protein in *Xenopus* oocyte nuclei (*1*, *2*). This protein is extremely acidic consisting of 23% glutamate and aspartate residues (*3*, *4*). Initially, the function of nucleoplasmin was postulated to be 2-fold: postfertilization decondensation of sperm chromatin by removal of the basic sperm protamines and assembly of nucleosomes by binding histones H2A and H2B and transferring them to DNA (*5*−*7*). Evidence suggests that nucleoplasmin may serve other functions as well. First, nucleoplasmin is present throughout oogenesis and not simply in the mature unfertilized egg as would be expected if only required postfertilization (*3*, *8*). Second, a nucleoplasmin-like protein has been identified in *Drosophila* somatic cells (*9*), suggesting that these highly acidic proteins are required for some function other than the decondensation of sperm chromatin as originally proposed. Finally, in the amphibian *Pleurodeles waltlii*, nucleoplasmin is found associated with lampbrush loops (*10*), which are regions of transcriptional activity, suggesting a role for nucleoplasmin in transcription.

The majority of eukaryotic DNA exists in the form of chromatin, and in order for transcription initiation to occur, some transcription factors must be able to gain access to DNA binding sites within chromatin. It has been demonstrated that nucleoplasmin stimulates the binding of transcription factors GAL-4, USF, and Sp1 to their cognate binding sites within nucleosomal DNA in vitro (*11*, *12*) and that nucleoplasmin functions in this capacity by removing the core histones H2A and H2B after transcription factor binding. In these studies, a heterologous system was used which mixed, yeast and mammalian trans-acting factors, *Xenopus* egg nucleoplasmin and HeLa cell histones. Although such a system provides a suitable model for examining the remodeling effects of nucleoplasmin, a more suitable homologous system would be required to determine whether this effect has any in vivo relevance.

The binding of *Xenopus* TFIIIA[1] to nucleosomal DNA has been demonstrated in vitro (*13*−*15*) and in one such case, it was shown that removal of H2A and H2B was required for TFIIIA binding (*14*). Thus, it was the object of this work to determine whether nucleoplasmin, isolated from *Xenopus* oocytes, could enhance the binding of *Xenopus* TFIIIA to nucleosomal 5S rRNA genes.

EXPERIMENTAL PROCEDURES

Protein Purification. Recombinant TFIIIA was purified from *Escherichia coli* cells harboring the expression plasmid pTF3 (*16*) while nucleoplasmin was purified from *Xenopus* eggs (*17*). Nucleosome core particles were isolated from chicken erythrocytes (*18*) as were purified histone octamers required for the supercoiling assay and nucleosome reconstitution (*19*). The purity of the proteins used in this study was verified by SDS-PAGE (*20*).

In Vitro Nucleosome Assembly. The ability of nucleoplasmin to assemble nucleosomes in vitro was tested by a supercoiling assay. Five hundred nanograms of histone octamers were incubated with or without 1 μg nucleoplasmin in 100 μL of 15 mM Tris, pH 7.5, 160 mM NaCl, 0.35 mM EDTA, 1 mM DTT, 1 mM β-mercaptoethanol, and 0.01% Nonidet P-40 for 45 min at room temperature. Following this, 0.5 μg of SV40 DNA in 5 μL of 50 mM Tris, pH 8, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, 20% glycerol, and 7 units/μL of topoisomerase I was added and incubation

[†] This work was supported by a grant of the Medical Research Council of Canada MT-13104 to J.A.

* To whom all correspondence should be addressed. Phone: (250) 721-8863. FAX: (250) 721-8855. E-mail: jausio@uvic.ca.

[‡] University of Victoria.

[§] Hokkaido University.

[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate, TFIIIA, transcription factor IIIA.

S0006-2960(97)02723-2 CCC: $15.00
Published on Web 01/15/1998

proceeded for 90 min at 37 °C. The reactions were centrifuged for 10 min at 15000*g*, and the supernatants were extracted with phenol/chloroform and ethanol precipitated. The different DNA topoisomers produced by deposition of histone octamers and subsequent supercoil relaxation were resolved by electrophoresis on a 2% agarose, 1× TPE (90 mM Tris-phosphate and 2 mM EDTA) gel and run at 4 V/cm for 16 h with buffer recirculation. The gel was ethidium bromide stained and the bands visualized under UV light.

Preparation and Labeling of 5S rRNA Gene Fragments. The two fragments of the *Xenopus laevis* oocyte 5S rRNA gene used in this study were designated Xlo(−77 → + 130) and Xlo(−32 → + 143) based on the position of the 5′ and 3′ termini in relation to the transcriptional start site. Xlo(−77 → + 130) positions a nucleosome at sites which permit TFIIIA binding whereas TFIIIA binding is blocked after reconstitution of a nucleosome on Xlo(−32 → + 143) (unpublished results). For use in this study, the fragments were excised from plasmids by *Hin*dIII/*Eco*RI digestion, 3′ end-labeled with Klenow and [α-^{32}P]dATP and purified by nondenaturing gel electrophoresis.

Nucleosome Reconstitution. The salt gradient dialysis method (*21*) and the exchange method were the two nucleosome reconstitution techniques used in this study. The salt gradient dialysis method, used to reconstitute full-length *Xenopus* oocyte 5S rRNA genes, used a DNA concentration of 100 μg/mL and a molar ratio of three histone octamers for every 5S rRNA gene repeat. The 720 bp full-length oocyte 5S rRNA gene repeat was isolated from *Hin*dIII digests of the plasmid pXlo8. For the exchange reaction, used to reconstitute the ∼185 bp 5S rRNA gene fragments, approximately 200 fmol of labeled DNA and 3 μg of cold, chicken erythrocyte nucleosome cores were incubated in 25 μL of 0.8 M NaCl, 50 mM Tris, pH 8, 1 mM β-mercaptoethanol, and 0.1 mM PMSF for 30 min at 37 °C. The nucleosomes were then incubated at 4 °C for 16 h, followed by stepwise dilution to 0.6 and 0.1 M NaCl by addition of 50 mM Tris, pH 8, and 0.1 mM PMSF after 30 min intervals at 4 °C.

TFIIIA Electrophoretic Mobility Shift Assay. TFIIIA binding reactions were performed with approximately 1 fmol of labeled nucleosomes or naked DNA in 10 μL of 20 mM Tris pH 7.5, 70 mM NaCl, 10 μM $ZnCl_2$, 6% glycerol, 0.1 mg/mL BSA, 2.5 mM DTT, 0.07% NP-40, 40 ng/μL poly(dI-dC)·poly(dI-dC) for 20 min at room temperature. The amount of nucleoplasmin and TFIIIA was as indicated. The binding reactions were loaded on a 0.75% agarose, 0.5× TB (45 mM Tris-borate) gel and run at 3.5 V/cm at room temperature. The gels were dried at 50 °C and autoradiographed. When free DNA was used as a binding substrate, 100 ng/μL poly(dI-dC)·poly(dI-dC) was added to the binding reaction.

Micrococcal Nuclease Digestion. Nucleosomes reconstituted on the 720 bp oocyte 5S rRNA gene repeat were adjusted to 1 mM $CaCl_2$ and digested with 20 units/mL micrococcal nuclease. Digestion was stopped and the DNA deproteinized by adjusting the solution to 5 mM EDTA, 0.25% SDS, and phenol/chloroform extracting. The digestion products were resolved on a 4% acrylamide gel (*22*).

In Vitro Transcription of Reconstituted 5S rRNA Genes. The HeLa nuclear transcription extracts were purchased from Promega Corp., and the transcriptions were performed as per manufacturer's instructions using 250 ng of template (either reconstituted nucleosomes or uncomplexed DNA). A $MgCl_2$ concentration of 2 mM was used and extracts were supplemented with 150 nM recombinant *Xenopus* TFIIIA.

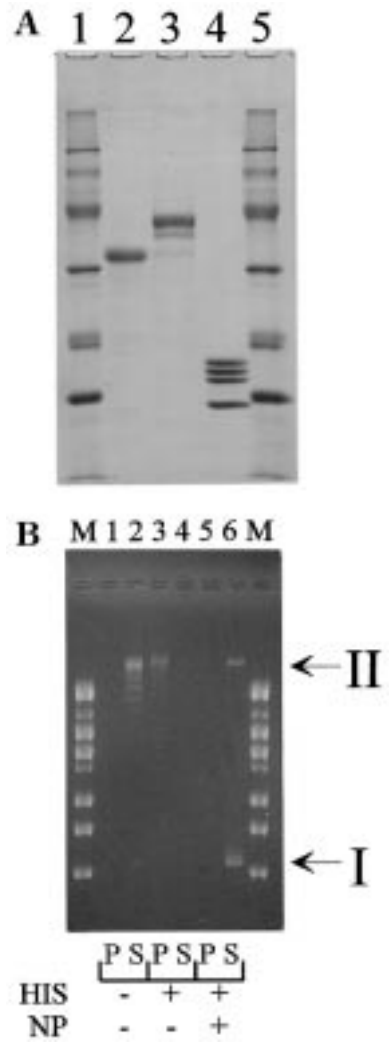

FIGURE 1: (A) SDS−PAGE analysis of the purified proteins used in this study. Lanes 1 and 5, low molecular weight protein markers (Bio-Rad); lane 2, nucleoplasmin purified from unfertilized *Xenopus* eggs; lane 3, recombinant *Xenopus* TFIIIA; lane 4, chicken erythrocyte core particle histones. (B) In vitro chromatin assembly capability of *Xenopus* nucleoplasmin. Five hundred nanograms of topoisomerase I treated, closed circular SV40 DNA (lane 2) was incubated with 0.5 μg of histone octamers previously mixed without (lanes 3 and 4) or with (lanes 5 and 6) 1 μg nucleoplasmin. After 90 min at 37 °C, the assembly reactions were centrifuged for 10 min at 15000*g* and both the pelleted (P) and supernatant (S) material were deproteinized and electrophoresed on a 2% agarose TPE gel to resolve the relaxed (II) or supercoiled (I) DNA forms.

RESULTS AND DISCUSSION

The proteins used in this study are shown in the SDS−PAGE in Figure 1A. It must be noted that, chicken erythrocyte histones and not *Xenopus* histones were used for this study. It is unlikely, however, that this effected the results due to the highly conserved nature of core histones (histones H3 and H4 share identical sequences in *Xenopus* and chicken). Furthermore, studies mapping nucleosome positions on reconstituted *X. laevis* 5S rRNA gene fragments showed similar results with both *Xenopus* and chicken erythrocyte histones (*23*). To demonstrate that the nucleo-

plasmin was functional with respect to nucleosome assembly activity, a supercoiling assay was performed and is shown in Figure 1B. In this assay, nucleosomes were mixed with closed circular SV40 DNA (one octamer for each 165 bp of DNA) at physiological concentrations of salt. Under these conditions, in the absence of nucleoplasmin, an insoluble complex formed (compare lanes 3 and 4) due to the electrostatic interactions between the DNA and histones. Nucleoplasmin served to circumvent this aggregation by binding histones and regulating the formation of a soluble complex (compare lanes 5 and 6) containing nucleosomes. The deposition of nucleosomes in the presence of topoisomerase I caused the DNA to become highly supercoiled as can be seen in lane 6.

Chen et al. (*11*) demonstrated that the histone binding protein, nucleoplasmin, is able to stimulate the binding of transcription factors GAL-4, USF, and Sp1 to their cognate binding sites within nucleosomal DNA. Nucleoplasmin is the most abundant protein in the nucleus of *Xenopus* oocytes, and thus, if it does play a role in enhancing transcription factor accessibility, this should also be observed with transcription factors present in *Xenopus* oocytes. Previous to this work, it was shown that, after reconstitution of 5S rRNA gene fragments into nucleosomes, the accessibility of TFIIIA for the intragenic promoter is dependent on nucleosome position (*13*, *15*, *23*). Furthermore, it was demonstrated that removal of histones H2A and H2B can overcome blockage of TFIIIA binding by nucleosomes (*14*). Thus, the initial objective was to determine whether nucleoplasmin, which preferentially removes histones H2A and H2B from nucleosomes (*11*), facilitates the binding of TFIIIA to the 5S rRNA gene after reconstitution of a nucleosome at a site which normally prevents TFIIIA binding. Figure 2A shows a TFIIIA electrophoretic mobility shift assay of the oocyte 5S rRNA gene fragment Xlo(−32 → + 143) complexed (lanes 1−10) or uncomplexed (lanes 11 and 12) into nucleosomes. The concentration of TFIIIA is indicated at the bottom of the gel, and the higher concentrations used in this study were sufficient to completely shift the corresponding uncomplexed DNA alone. As can be seen in lanes 1−5, a nucleosome on this gene fragment blocked TFIIIA binding. Upon addition of nucleoplasmin to the binding reaction, a nucleosomal shift by TFIIIA was still absent (lanes 6−10), indicating nucleoplasmin was unable to circumvent the nucleosomal blockage of TFIIIA binding to this gene fragment. The results suggest that, despite the previously reported nucleosome remodeling ability of nucleoplasmin, the binding of TFIIIA to nucleosomal DNA could not be facilitated by this protein.

One possible explanation for the discrepancy between our results and those of Chen et al. (1994) is that in this previous study, a small amount of transcription factor binding was already evident in the absence of nucleoplasmin, and the addition of nucleoplasmin served to greatly increase this binding. In our study, however, no binding of TFIIIA to nucleosomal Xlo(−32 → + 143) DNA was evident, and thus, we repeated the TFIIIA electrophoretic mobility shift assay using the 5S rRNA gene fragment Xlo(−77 → + 130) instead. This fragment positions nucleosomes in a manner which permits TFIIIA binding but at much higher concentrations of TFIIIA than is required for binding of naked DNA alone. We were thus testing whether nucleoplasmin could

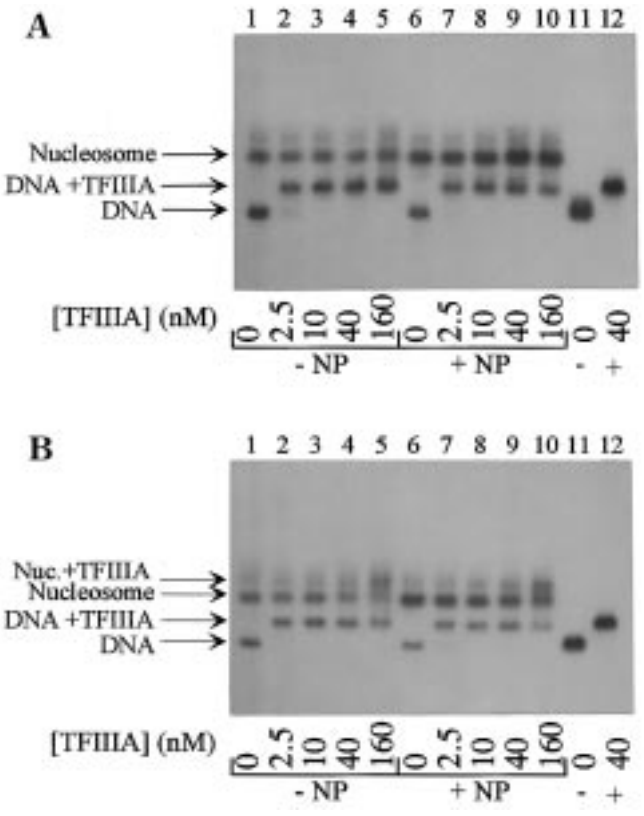

FIGURE 2: Agarose gel electrophoresis analysis of the effect of nucleoplasmin on the binding of TFIIIA to nucleosomal *Xenopus laevis* 5S rRNA gene fragments. (A and B) Nucleosomes (1 fmol) reconstituted with chicken erythrocyte histones on Xlo(−32 → + 143) (A) or Xlo(−77 → + 130) (B) in the absence (lanes 1−5) or presence (lanes 6−10) of 150 ng of *Xenopus* nucleoplasmin and increasing amounts of TFIIIA. Lanes 11 and 12, the corresponding uncomplexed DNA in the absence (−) or presence (+) of TFIIIA. The samples were incubated for 20 min at room temperature before loading on agarose gels.

enhance the binding of TFIIIA to nucleosomal DNA. The results, shown in Figure 2B, demonstrate that a 160 nM concentration of TFIIIA partially shifted Xlo(−77 → + 130) after reconstitution into nucleosomes (lane 5). If nucleoplasmin was able to remodel this nucleosome in a manner which facilitated TFIIIA binding, this shift should be evident at lower concentrations of TFIIIA, which is not the case (lanes 6−10). This demonstrates that nucleoplasmin was unable to enhance the binding of TFIIIA to nucleosomal DNA.

Previously, it was shown that deposition of nucleosomes onto plasmids containing 5S rRNA genes repressed 5S rRNA transcription (*24*, *25*). Similar plasmids, reconstituted with physiological levels of histones H3 and H4 alone, are permissive to 5S rRNA transcription (*26*). We took advantage of these results to test whether nucleoplasmin, by selectively removing H2A and H2B, could enhance transcription of nucleosomal 5S rRNA genes. To this end, nucleosomes were reconstituted onto a full-length oocyte 5S rRNA gene repeat (720 bp DNA fragment containing the 120 bp 5S rRNA coding sequence and the nontranscribed spacers) by a salt gradient dialysis method, and these genes were transcribed in HeLa nuclear extracts supplemented with TFIIIA and increasing amounts of nucleoplasmin. Figure 3A is a micrococcal nuclease time course digestion which demonstrates that nucleosomes were present on the genes,

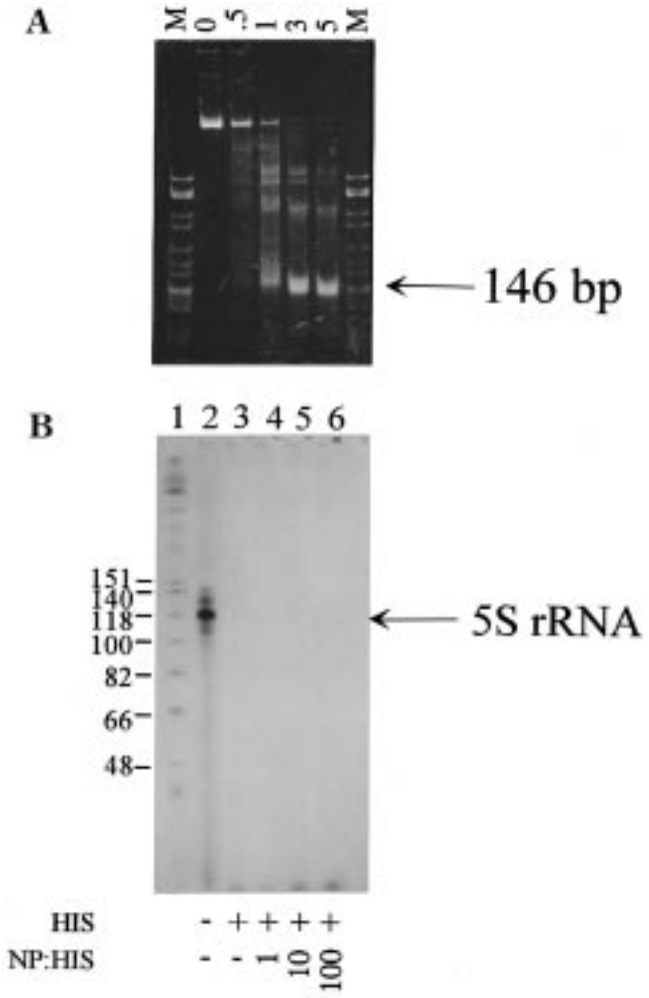

FIGURE 3: The effect of nucleoplasmin on the transcription of reconstituted *Xenopus laevis* oocyte 5S rRNA genes. (A) Micrococcal nuclease digestion of nucleosomes reconstituted on the *Xenopus laevis* oocyte gene. Digestions were carried out at a nucleosome concentration of 0.1 mg/mL (DNA weight) and enzyme concentration of 20 units/mL for the minutes indicated on the top of each lane. The resulting DNA fragments were deproteinized and electrophoresed on a 4% nondenaturing gel. (M) *Hha*I cut pB*R*322. (B) Approximately 250 ng of an oocyte 5S rRNA gene either uncomplexed (lane 2) or reconstituted with histones isolated from chicken erythrocytes (lanes 3−6) were transcribed in the absence (lanes 2 and 3) or presence (lanes 4−6) of increasing amounts of nucleoplasmin as shown. The transcriptions were performed in HeLa nuclear extracts supplemented with 150 nM recombinant *Xenopus* TFIIIA and 2 mM $MgCl_2$. Transcripts were analyzed by denaturing polyacrylamide gel electrophoresis (8% acrylamide and 8.3 M urea in 1× TBE). Lane 1, Klenow end-labeled *Hinf*I cut ϕX174 DNA (sizes of marker fragments shown as number of nucleotides).

as is indicated by 146 bp micrococcal nuclease resistant fragments. Results of transcription studies, shown in Figure 3B, indicate that nucleosomes reconstituted on the oocyte gene repeat repressed 5S rRNA transcription (lane 3). Nucleoplasmin did not circumvent this repression even at ratios of 100 molecules of nucleoplasmin for every histone octamer present (lanes 4−6).

The results of our analysis showed that nucleoplasmin did not enhance the binding of TFIIIA to nucleosomal DNA. Furthermore, nucleoplasmin was unable to enhance transcription of 5S rRNA from nucleosomal templates. These results are not surprising considering, although nucleoplasmin has been shown to specifically bind histones H2A and H2B, it is unable to release these histones from chromatin during chromosome remodeling in oocyte extracts (*27*).

ACKNOWLEDGMENT

We are very grateful to Dr. Nik Veldhoen and Dr. Paul Romaniuk for assistance in the preparation of TFIIIA.

REFERENCES

1. Mills, A. D., Laskey, R. A., Black, P., and De Robertis, E. M. (1980) *J. Mol. Biol. 139*, 561−568.
2. Krone, G., and Franke, W. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1034−1038.
3. Burglin T. R., Mattaj, I. W., Newmeyer, D. D., Zeller, R., and DeRobertis, E. M. (1987) *Genes Dev. 1*, 97−107.
4. Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S., and Laskey, R. A. (1987) *EMBO J. 6*, 69−74.
5. Philpott, A., and Leno, G. H. (1992) *Cell 69*, 759−767.
6. Laskey, R. A., Mills, A. D., Philpott, A., Leno, G. H., Dilworth, S. M., and Dingwall, C. (1993) *Philos. Trans. R. Soc. London Biol. 339*, 263−269.
7. Katagiri, C., and Ohsumi, K. (1994) *Int. J. Dev. Biol. 38*, 209−216.
8. Litvin, J., and King, M. L. (1988) *Development 102*, 9−21.
9. Ito, T., Tyler, J. K., Bulger, M., Kobayashi, R., and Kadonaga, J. T. (1996) *J. Biol. Chem. 271*, 25041−25048.
10. Moreau, N., Angelier, N., Bonnanfant-Jais, M. L., Gounon, P., and Kubisz, P. (1986) *J. Cell. Biol. 103*, 683−690.
11. Chen, H., Li, N., and Workman, J. L. (1994) *EMBO J. 13*, 380−390.
12. Walter, P. P., Owen-Hughes, T. A., Cote, J., and Workman, J. L. (1995) *Mol. Cell. Biol. 15*, 6178−6187.
13. Rhodes, D. (1985) *EMBO J. 4*, 3473−3482.
14. Hayes, J. J., and Wolffe, A. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1229−1233.
15. Lee, D. Y., Hayes, J. J., Pruss, D., and Wolffe, A. P. (1993) *Cell 72*, 73−84.
16. Veldhoen, N., You, Q. M., Setzer, D. R., and Romaniuk, P. J. (1994) *Biochemistry 33*, 7568−7575.
17. Rice, P., Garduno, R., Itoh, T., Katagiri, C., and Ausio, J. (1995) *Biochemistry 34*, 7563−7568.
18. Ausio, J., Dong, F., and van Holde, K. E. (1989) *J. Mol. Biol. 206*, 451−463.
19. Hansen J. C., Ausio, J., Stanik, V. H., and van Holde, K. E. (1989) *Biochemistry 28*, 9129−9136.
20. Laemmli, U.K. (1970) *Nature 227*, 680−685.
21. Tatchell, K., and van Holde, K. E. (1977) *Biochemistry 16*, 5295−5303.
22. Yager, T. D., and van Holde, K. E. (1984) *J. Biol. Chem. 259*, 4212−4222.
23. Gottesfeld, J. M. (1987) *Mol. Cell. Biol. 7*, 1612−1622.
24. Gottesfeld, J. M., and Bloomer, L. S. (1980) *Cell 21*, 751−760.
25. Stunkel, W., Kober, I., Kauer, M., Taimor, G., and Seifart, K. H. (1995) *Nucleic Acids Res. 23*, 109−116.
26. Tremethick, D., Zucker, K., and Worcel, A. (1990) *J. Biol. Chem. 265*, 5014−5023.
27. Dimitrov, S., and Wolffe, A. P. (1996) *EMBO J. 15*, 5897−5906.

BI972723+