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Histone Variant H2A.Z Inhibits Transcription in Reconstituted Nucleosomes†

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ABSTRACT: The existence of histone nonallelic variants has been known for more than 30 years, but only recently have we acquired significant insights into their functions. Nucleosomes containing histone variants are nonrandomly distributed in genomes and may impart different biological functions to the relevant chromatin regions. We have used the model T7 RNA polymerase to transcribe reconstituted nucleosomes containing either canonical human recombinant histones or two histone variants, H2A.Z or H3.3, whose presence has been associated with active transcription. Remarkably, in contrast to canonical and H3.3-containing nucleosomes, H2A.Z-containing nucleosomes were refractive to transcription, with residual levels of transcription determined by the sequence of the underlying DNA template. To our knowledge, this is the first example of a nucleosome that is intrinsically untranscribable.

Gene expression in eukaryotes is regulated, in part, through changes in chromatin structure (*1*). These changes are brought about by the activities of ATP-dependent chromatin remodeling complexes, by posttranslational histone modifications, by changing histone stoichiometry in the nucleosome (*2*), and/or by replacing canonical histones with nonallelic histone variants (*3*). These "replacement" variants are deposited onto chromatin throughout the cell cycle, replacing the resident canonical histones that are incorporated during S phase. At least three variants have been linked to transcriptional activity: H3.3, H2A.Z, and H2A.Bbd (*1*, *4*). There is a common intriguing feature of H2A.Z and H3.3: these variants may be markers for chromatin regions in flux. Thus, H3.3-containing nucleosomes are enriched over *cis*-regulatory boundary elements in the *Drosophila* genome, suggesting that chromatin structure is in constant flux, probably as part of a mechanism to keep *cis* elements exposed to factor binding (*5*). H3.3 also marks actively transcribed regions, where nucleosomes are constantly disassembled and reassembled (*6*, *7*). H2A.Z is also present in chromatin regions in flux. Rando's group (*8*) demonstrated that yeast nucleosomes that turn over rapidly ("hot" nucleosomes) are highly enriched for H2A.Z; the two H2A.Z-bearing nucleosomes that surround transcription start sites genome-wide (*9*) are among the hottest. Thus, nucleosomes containing H2A.Z or H3.3 are clearly markers for chromatin regions in flux. The question then arises: is the presence of these variants just a reflection of high nucleosome turnover (independently of the biological context), or do they possess certain characteristics that directly affect the behavior of nucleosomes? This question is especially relevant to transcription elongation, because both H2A.Z and H3.3 are found in the bodies of transcribed genes (*4*, *7*, *10*, *11*). Some calculations from genome-wide data in human cells provide an estimate of overall content of H2A.Z-containing nucleosomes in transcribed regions of genes as ~55% of the total (Tolstorukov, personal communication).

Here we focus on the transcriptional behavior of reconstituted nucleosomes containing human recombinant canonical histones or histone variants H2A.Z and H3.3 and a combination thereof. Double-variant H2A.Z/H3.3 nucleosomes exist *in vivo* and have been reported to be less stable than canonical nucleosomes or nucleosomes containing only H3.3 (*12*). On specific genes and genome-wide, H2A.Z/H3.3 nucleosomes mark active promoters and other regulatory regions, but they are also found at the beginning of transcribed regions (*12*, *13*). We used two distinct DNA fragments as reconstitution substrates and the single-subunit T7 RNA polymerase as a model polymerase. Phage polymerases (T7 and SP6) have been extensively used in studies of chromatin transcription since they are excellent probes for aspects of transcription that are determined by the properties of the nucleosomes themselves and not by the nature of the particular RNAP (*14*−*17*). Importantly, most effects of a transcribing polymerase on nucleosomes are expected to result from the mechanics of transcription on DNA, i.e., local denaturation, translocation, and rotation of the DNA with respect to the catalytic center of the RNAP, creating torsion in topologically constrained templates, etc.

EXPERIMENTAL PROCEDURES

Construction of DNA Templates Carrying the T7 RNAP Promoter Sequence. (*A*) *208 T7 Template*. The DNA template used for PCR[1] was plasmid p2T7/T3-208-18, a gift of V. Jackson, Medical College of Wisconsin, containing the T7 promoter and 18 tandem repeats of the 208-bp 5S gene of *Lytechinus variegates* (*18*). The primers used were pT7208F24, 5′-CTC GAG AAT TCG GTA TTC CCA GGC-3′, and pT7208R24, 5′-CTC GAG ACC CAA GCT TCC CGA GTA-3′. The PCR amplification was done using Taq polymerase (New England BioLabs, Ipswich, MA) at T_E = 58 °C. The monomer 208-T7 band was extracted from an agarose gel using the QIAEX

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[1]Abbreviations: PCR, polymerase chain reaction; AUT, acetic acid−urea−Triton X-100; LH, linker histone; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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II gel extraction kit (QIAGEN Sciences, Maryland, MD), then ligated into a PCR4 TOPO vector (Invitrogen, Carlsbad, CA), and finally transformed into XLI Blue competent cells (Stratagene, La Jolla, CA) using the manufacturer's protocol. The 208-T7 DNA insert was cut out of the plasmid with *Xho*I restriction enzyme (New England BioLabs, Ipswich, MA) and dissolved in 10 mM Tris-HCl, pH 8.0.

(*B*) *m* (*Modified*) *GUB-T7 Template*. GUB (*19*, *20*) DNA was modified at a few base pairs by PCR using four different reverse primers in four steps to prepare mGUB-T7. The PCR product after the first PCR step was used as template in the subsequent step, after purifying the product using the QIAquick PCR purification kit (QIAGEN Sciences, Maryland, MD). Primers used for the first step were GUBT7-for, 5′-CTA GAG GAT CCT CTA GAC GGA-3′, and mGUB-T7-rev1 (85−135), 5′-GAT ATG TTT AAA TTG GAG AGT AGT CCA AGG AAC GAA GCA AGC AGG ATC TAC G-3′. The 135-bp PCR product was used as template for the second step, using primers GUBT7-for, 5′-CTA GAG GAT CCT CTA GAC GGA-3′, and mGUB-T7-rev2 (111−165), 5′-AAT ATT TCG CGA AGG CCA CCC GGG CAG CTG GAT ATG TTT AAA TTG GAG AGT AGT C-3′. For the third step, the primers were GUBT7-for, 5′-CTA GAG GAT CCT CTA GAC GGA-3′, and mGUB-T7-rev3 (148−190), 5′-AGG GAG AGA AGA GTG TAG TAG TAG GAA TAT TTA GAG AAG GCC A-3′, and primers for the fourth step were GUBT7-for, 5′-CTA GAG GAT CCT CTA GAC GGA-3′, and mGUB-T7-rev4 (166−216), 5′-GAT GCT GAG GTA ATA CGC ATC ACT ATA GGG AGA GAA GAG TGT AGT AGT AGG-3′. The PCR amplification was done using Taq polymerase at T_E = 67, 67, 66.6, and 67.7 °C, respectively. The 216-bp-long PCR product was purified using the QIAquick PCR purification kit (QIAGEN Sciences, Maryland, MD), then ligated into a pUC vector, and transformed into XLI Blue competent cells (Stratagene, La Jolla, CA) using the manufacturer's protocol. The GUB DNA insert was cut out of the plasmid by digesting with *Eco*RI and *Pst*I restriction enzymes (New England BioLabs, Ipswich, MA) and dissolved in 10 mM Tris-HCl, pH 8.0.

Human Recombinant Histone Expression, Purification, and Analysis. The coding sequences for canonical human histones H2A, H2B, H3, and H4, as well as for H2A.Z, were cloned into the pET-22b expression vector (expression clones provided by Dr. Fukui, Osaka University, Japan). We have recloned *Drosophila* H3.3 (original clone provided by S. Henikoff) into the bacterial expression vector pET-3 (*Drosophila* and human H3.3 are identical). The recombinant histones were overexpressed in Rosetta (DE3) pLysS cells, purified by tandem ion-exchange chromatography (Q-Sepharose/SP-Sepharose; Amersham Biosciences, Piscataway, NJ), and checked on AUT−PAGE (5% acetic acid, 5.25 M urea, 5 mM Triton X-100, 10.5% PAGE) (left panel) and SDS−PAGE (right panel) as described (*21*) (Figure 2A). Octamers were reconstituted by dialyzing from 8 M guanidinium chloride to 2 M NaCl (*22*), purified on a Superdex (Amersham Biosciences) column, and checked on 15% SDS−PAGE (*23*) (Figure 2B).

Nucleosome Reconstitution and Analysis of Reconstituted Nucleosomes on Native Agarose and Polyacrylamide Gels. Nucleosomes were reconstituted by the salt-jump method (*24*, *25*) and analyzed on 1% native agarose gels (Figure 6A) or 5.5% native PAGE gels (Figure 2C).

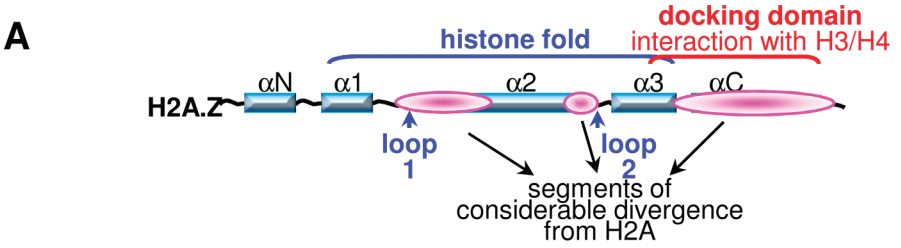
Restriction Nuclease Accessibility Assay. To check if the T7 promoter is not occluded by a nucleosome in the 208-T7 H2A.Z reconstitute, 2 μg of nucleosomes was digested with different restriction endonucleases (*Dra*I and *Hin*fI) in commercially provided buffers (New England BioLabs, Ipswich, MA) for 2 h at 37 °C. Digested samples were phenol−chloroform extracted, ethanol precipitated, and analyzed on 12% PAGE in 1× TBE, pH 8.3 [10.8 g of Tris base (Roche Diagnostics, Indianapolis, IN), 5.5 g of boric acid (Fisher Scientific, Fair Lawn, NJ), and 0.75 g of sodium EDTA (Fisher Scientific, Fair Lawn, NJ) per liter of ultrapure water].

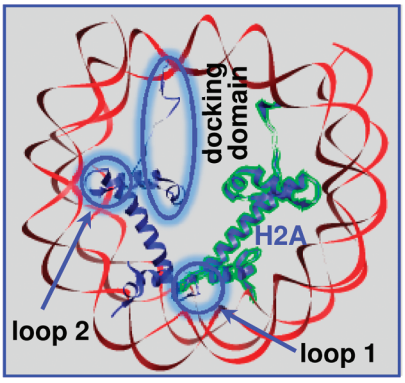
In Vitro Transcription. *In vitro* transcription was performed at 37 °C using the T7 Ampliscribe transcription kit (Epicenter Biotechnologies, Madison, WI) according to the manufacturer's protocol. Alternatively, T7 RNAP (Epicenter Biotechnologies) was used for transcription in either homemade transcription buffer (25 mM HEPES−NaOH, pH 7.9, 5 mM $MgCl_2$, 0.1% Tween 20, 0.1 mg/mL BSA, 20 mM NaCl, and 3 mM 2-mercaptoethanol) containing 7.5 mM NTPs or the Epicenter Biotechnologies 5× transcription buffer (1× is 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$,10 mM NaCl, and 2 mM spermidine) with practically the same results. Following transcription, the products were treated with 2 units of DNase I (RNase-free) (Epicenter Biotechnologies, Madison, WI) for 15 min at 37 °C to remove the template DNA. RNA was purified by phenol−chloroform−2-propanol extraction and ethanol precipitation. Samples were mixed with an equal volume of 2× loading buffer (7 M urea, 50 mM EDTA, 0.01% xylene cyanol, and 0.01% bromophenol blue). The reaction mixtures were denatured at 100 °C for 5 min and fractionated on 12% urea−PAGE gels.

RESULTS

Histone variants H2A.Z and H3.3 differ from their canonical counterparts to a different degree. H2A.Z possesses numerous amino acid substitutions when compared to the major H2A subtype; H3.3 differs from H3 in only four amino acid residues. Figure 1A,B illustrates the location of the primary sequence differences with respect to the secondary structure of histone H2A.Z and the crystal structure of the nucleosome core particle (*26*, *27*).

H2A.Z-Containing Nucleosomes Fail To Be Transcribed by a Model RNA Polymerase. We reconstituted nucleosomes on two different nucleosome positioning sequences, the widely used 208-bp 5S rDNA from sea urchin *L. variegates* (*28*) and a slightly modified GUB sequence [mGUB was originally constructed for experiments aimed at addressing the fate of canonical nucleosomes during transcription elongation and differs from classical GUB (*19*) in only 10 positions]. Figure 1C presents the two sequences and the reported major nucleosome positions. Note that we have added the T7 RNAP promoter sequence onto the 3′ end of the fragments. These sequences were reconstituted into nucleosomes using four distinct sets of human recombinant histone octamers: canonical $(H2A/H2B/H3/H4)_2$, H2A.Z-containing $(H2A.Z/H2B/H3/H4)_2$, H3.3-containing $(H2A/H2B/H3.3/H4)_2$, or containing both H2A.Z and H3.3 $(H2A.Z/H2B/H3.3/H4)_2$ (Figure 2A,B). The success of reconstitution was assessed by either native agarose gels (the 208-T7 example is presented in Figure 6A) or nondenaturing PAGE gels (Figure 2C). To make sure that transcription occurred on the nucleosomal templates and not on the naked DNA usually present in nucleosome preparations, the transcription reactions were performed on preparations that did not contain more than





C

208-T7 (5S rDNA + T7 promoter, 317 bp, CG 52%)

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5′ CTCGAGAATT CGGTATTCCC AGGCGGTCTC CCATCCAAGT ACTAACCGAG
   CCCTATGCTG CTTGACTTCG GTGATCGGAC GAGAACCGGT ATATTCAGCA
   TGGTATGGTC GTAGGCTCTT GCTTGATGAA AGTTAAGCTA TTTAAAGGGT
   CAGGGATGTT ATGACGTCAT CGGCTTATAA ATCCCTGGAA GTTATTCGTT
   GGAATTCCTC GGGCAGCGTT GGGTCCTGGC CACGGGTGCG AGCTCGGTAC
   CCGGGGATCC TCTAGACTCG GGTCTCCCTA TAGTGAGTCG TATTACTCGG
   GAAGCTTGGG TCTCGAG 3′          +1
```

mGUB-T7 (modified GUB + T7 promoter, 216 bp, CG 50.4%)

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5′ CTAGAGGATC CTCTAGACGG AGGACAGTCC TCCGGTTACC TTCGAACCAC
   GTGGCCGTCT AGATGCTGAC TCATTGTCGA CACGCGTAGA TCTGCTTGCT
   TCGTTCCTTG GACTACTCTC CAATTTAAAC ATATCCAGCT GCCCGGGTGG
   CCTTCGCGAA ATATTCCTAC TACTACACTC TTCTCTCCCT ATAGTGAGTC
   GTATTACCTC AGCATC 3′                      +1
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FIGURE 1: Structural differences between canonical histone H2A and its replacement variant H2A.Z. (A) Secondary structures of the H2A.Z histone molecule: blue rectangles, α-helices; brackets encompass the histone fold and the docking domain in H2A; pink ovals, locations of the primary sequence with considerable divergence between canonical H2A and H2A.Z. (B) Portions of the crystal structure of the nucleosome core particle, containing, for clarity, only nucleosomal DNA and two molecules of H2A.Z. The major regions of divergence are circled in blue. The models were created from crystal structure coordinates (NCBI PDB code 1F66) using UCSF Chimera. (C) Nucleotide sequences of the DNA fragments (template strands) used for nucleosome reconstitution. The DNA sequences that wrap around canonical octamers are boxed and set in green; the T7 RNAP promoter is in red, with +1 marking the transcription start site. The *Dra*I and the *Hin*fI sites on 208-T7 are in blue, bold, and underlined. The dyad axes are in purple, bold, and italics. The nucleotides that differ between mGUB and classical GUB (*19*) are set in blue, bold.

5% of naked DNA; such preparations were obtained after careful optimization of the histone/DNA ratios for reconstitution (Figure 2C)].

To assess the effect of variant nucleosomes on transcription, we performed T7 RNAP-mediated transcription experiments and analyzed the RNA transcripts following removal of the template with DNase I. Under our transcription conditions, the integrity of the nucleosome was not compromised (Figure 3). To our utmost surprise, the 208-T7 nucleosome containing either H2A.Z or the H2A.Z/H3.3 hybrid failed to be transcribed at all. Under the same conditions, the control canonical nucleosome and the H3.3-containing particle showed robust transcription (Figure 4A). The presence of H2A.Z was dominant over that of H3.3, since the hybrid particles were not transcribed (exactly as the particles containing only H2A.Z). This result was persistent even under slightly different experimental conditions (varying NTP concentrations, duration of reaction, slightly different transcription buffers). In canonical or H3.3 nucleosomes T7 RNAP paused at intrinsic DNA sequence-dependent pause sites, in agreement with results obtained with nucleosomes reconstituted on other naturally occurring sequences (*29*, *30*).

H2A.Z Does Not Directly Inhibit T7 RNAP. The total lack of transcription in the H2A.Z-containing nucleosomes could have a trivial explanation: that H2A.Z directly inhibits the enzymatic activity of T7 RNAP. That this was not the case

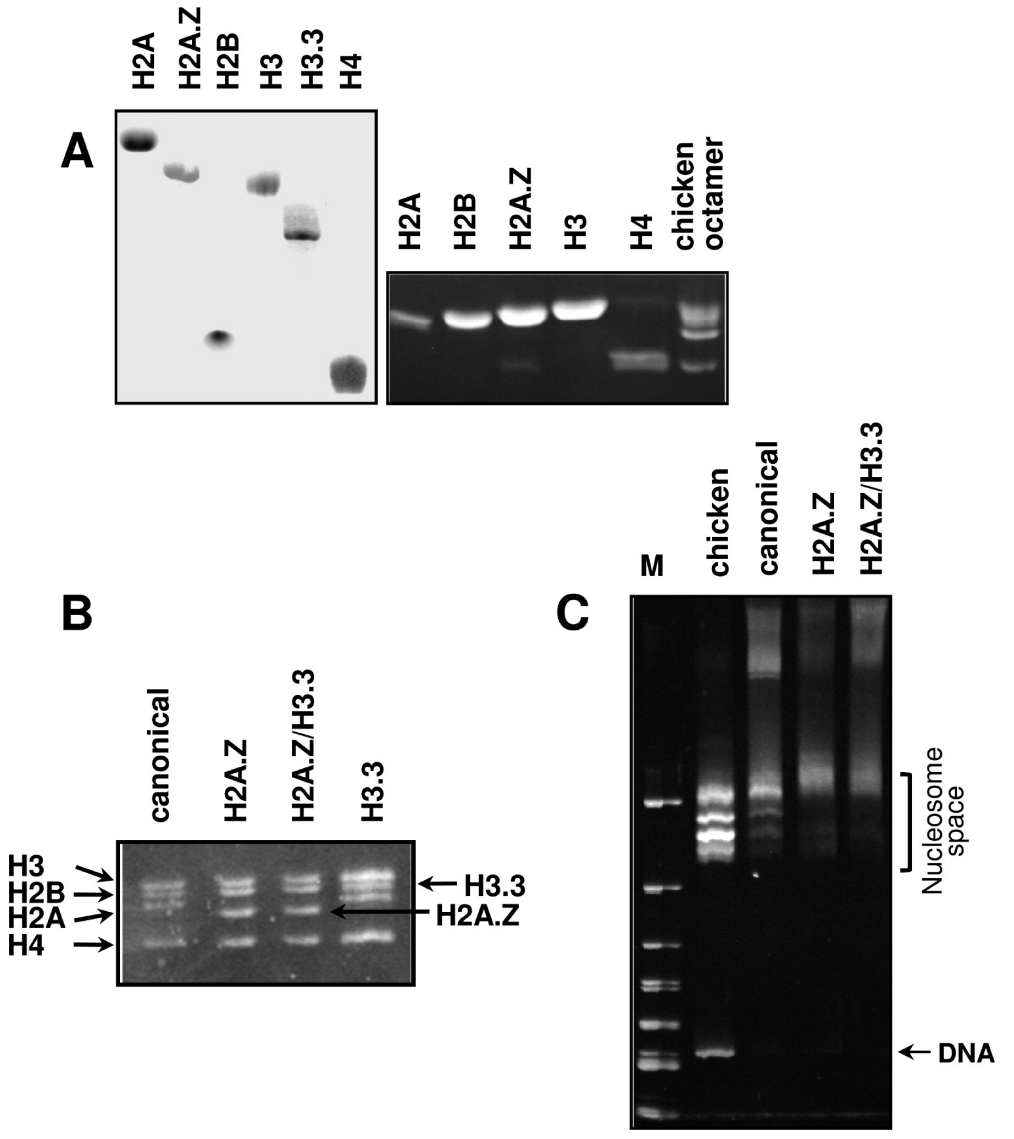

FIGURE 2: Human recombinant histones used for nucleosome reconstitution and analysis of the reconstituted particles on native PAGE gels. (A) Human recombinant histones used for octamer reconstitution, analyzed by Triton/acid−urea electrophoretic gels (left panel) and SDS gels (right panel). (B) Assembled octamers analyzed by SDS gels. (C) Band-shift analysis of nucleosome reconstitutions on the 208-T7 317-bp sequence on 5.5% native PAGE gels. Lane marked "chicken" stands for particles reconstituted with histone octamers extracted from chicken erythrocytes (*51*).

was obvious by the presence of very short transcripts on gels run for shorter times (Figure 5A). We also performed reactions in which canonical promoter-containing nucleosomes were transcribed in the presence of equal amounts of promoterless H2A.Z particles (Figure 5B). The gel indicates that some inhibition of transcription on the canonical particles was inflicted by the presence of their H2A.Z-containing counterparts. This slight inhibitory effect could also explain the relative paucity of the shorter transcripts which originate from the naked portion of the template before the polymerase reaches the nucleosome (Figure 5A). Importantly, however, it is clear that the degree of this inhibition is such that it cannot explain the total inhibition that we see in the H2A.Z-containing particles.

H2A.Z Inhibits Transcription on Nucleosomes Reconstituted on an Unrelated DNA Nucleosome Positioning Sequence. To see whether the behavior of H2A.Z-containing nucleosomes was not just a peculiarity of the 208-T7 reconstitution substrate, we used the totally unrelated mGUB-T7 sequence for reconstitution. mGUB-T7 was also transcribed to a very low level when assembled with H2A.Z (Figure 4B). Hardly any full-length transcripts were produced, and the residual transcripts appeared to arise from pausing/stalling of the polymerase at DNA sequence-dependent sites (compare transcript patterns on H2A.Z-containing nucleosomes with patterns on naked DNA), as was the case for canonical and H3.3-containing nucleosomes. In addition, the reaction was much less efficient than that on canonical particles (Figure 4B). Transcription through the canonical particles did go to completion (full-length transcripts accumulated with time); however, as expected (*29*, *31*), the efficiency of transcription through the nucleosome was much less than that on the naked DNA template (Figure 4B).

The T7 RNAP Promoter in the 317-bp 208-T7 Reconstitute Is Accessible to Polymerase Binding. The nucleosome position depicted on the 208-T7 template in Figure 1C has been determined on a 208 fragment 208 bp in length (*32*); a similar major position has been found on a somewhat longer (256-bp) fragment of the same sequence (*33*). It is theoretically possible

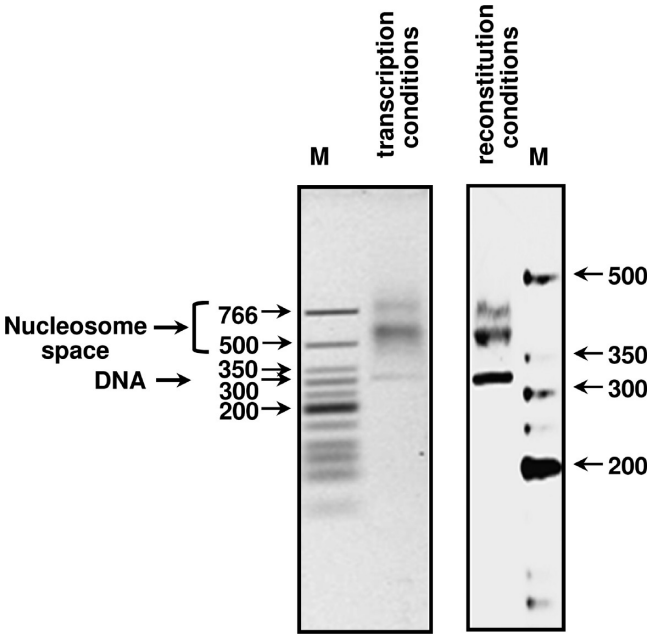

FIGURE 3: Analysis of the integrity of the nucleosome under transcription reaction conditions. Left panel: Canonical nucleosomes reconstituted on the 208-T7 317-bp sequence and run on 1% agarose gels under transcription reaction conditions. M is low molecular weight DNA ladder (New England BioLabs, Ipswich, MA). Right panel: A representative nucleosome reconstitution reaction under reconstitution conditions, analyzed on 1.5% agarose gel. Note that the relative disposition of the two nucleosomal bands and the naked DNA is the same in the right and left panels; the difference in the electrophoretic mobility of the particles with respect to the marker DNA fragments is due to the different percentage of the gel used.

that the longer 317-bp fragment can accommodate two closely situated nucleosomes, occluding the promoter and preventing T7 RNAP binding. The promoter is evidently free in the majority of canonical and H3.3-containing nucleosomes, because they are efficiently transcribed; however, H2A.Z might shift nucleosome positions (*34*) or redistribute nucleosomes in the already available position space (*35*).

To address this possibility, we analyzed nucleosomes reconstituted on the 317-bp 208-T7 sequence by native agarose (Figure 6A) or polyacrylamide gels (Figure 6B). In agarose gels two retarded bands would be expected if the sequence accommodates two nucleosomes. Indeed, both H2A.Z-containing and canonical nucleosome preparations exhibited two retarded bands on agarose gels, corresponding to fragments containing one and two nucleosomes, respectively, in a ratio of 2:1 (Figure 6A). The polyacrylamide gels resolve alternative octamer positions along the DNA sequence, with a centrally positioned particle possessing the lowest electrophoretic mobility (*36*). As Figure 6B shows, the distribution of nucleosome positions on the canonical and H2A.Z-containing 317-bp 208-T7 reconstitutes differed only very slightly. Thus, the two nucleosome populations, canonical and H2A.Z-containing, that differ so drastically in their transcriptional efficiencies did not differ significantly in their gross structure.

To directly assess whether the T7 promoter is not occluded by a nucleosome in the 208-T7 H2A.Z reconstitute, we performed restriction nuclease accessibility assays. We compared the levels of digestion of canonical and H2A.Z-containing nucleosomes with two restrictases: *Dra*I, whose recognition site is in the middle of the major nucleosome position (and is expected to be protected from digestion), and *Hin*fI, which cleaves the promoter region. Whereas the *Dra*I site is fully protected in both particles (as expected), the *Hin*fI site is digested to completion in the naked DNA preparation only (Figure 6C). In canonical and H2A.Z-containing reconstitutes, about two-thirds of the particles were digested, whereas about one-third was protected, in agreement with the patterns seen in Figure 6A, where the majority of the templates contained only one nucleosome, and a smaller portion contained two nucleosomes. The restriction nuclease digestions were performed on preparations that did not contain more than 5% of naked DNA; these were the preparations used for the transcription experiments. Thus, about 70% of both the canonical *and* H2A.Z particles had the T7 RNAP promoter accessible for polymerase binding. Thus, the lack of transcription in the H2A.Z-containing nucleosome is *not* a consequence of occluding the promoter with a nucleosome.

DISCUSSION

As a whole, our results with T7 RNAP indicate that nucleosomal transcription is strongly dependent on the presence of histone variant H2A.Z. All other conditions being equal, the H2A.Z-containing nucleosomes are refractive to transcription (Figure 4). The nucleotide sequence of the H2A.Z-containing particle fine-tunes the final transcriptional outcome.

Why were we surprised by the severe inhibition of transcription in H2A.Z-containing nucleosomes? First, we are unaware of *any* nucleosome that would be intrinsically untranscribable. The very fact that we found one was a big surprise.

Second, the relationship between the presence and the level of H2A.Z-containing nucleosomes and transcription is extremely complex and obviously species-specific (*4*). Whereas in yeast there seems to be an inverse correlation between the two (*4*), the situation in multicellular eukaryotes (*Drosophila* and humans) is, in general, exactly the opposite: H2A.Z levels actually correlate with transcription (*10*, *37*). Recent genome-wide localization studies in murine ES cells revealed that 93% of the H2A.Z-enriched promoters were also occupied by polycomb group (PcG) proteins, which are known to maintain target genes in a silent state (*38*). Moreover, H2A.Z and PcG occupancy was interdependent on these promoters, although no direct interaction between the two was observed (*38*). With the underlying mechanisms unclear, it is not possible to make any inferences on a direct involvement of H2A.Z in transcription silencing. Another recent study showed cooperation of H2A.Z with heterochromatin factors in suppression of potentially deleterious antisense RNAs (*39*). Among the numerous possible mechanisms discussed, the authors suggested that H2A.Z nucleosomes might directly obstruct Pol II progression. Our results with the model RNA polymerase support such a notion; however, it needs to be directly addressed in *in vitro* experiments (see below).

It must be noted that all of these studies address H2A.Z occupancy mainly in promoter gene regions, around transcription start sites; the relationship between H2A.Z presence and transcription elongation has not been addressed in detail until very recently (see below). Thus, based on the high species specificity of H2A.Z transcriptional effects, there was no a priori expectation that the H2A.Z-containing particle by itself will be refractory to transcription.

Several very recent papers are consistent with our results; moreover, our results may provide an explanation for the data in

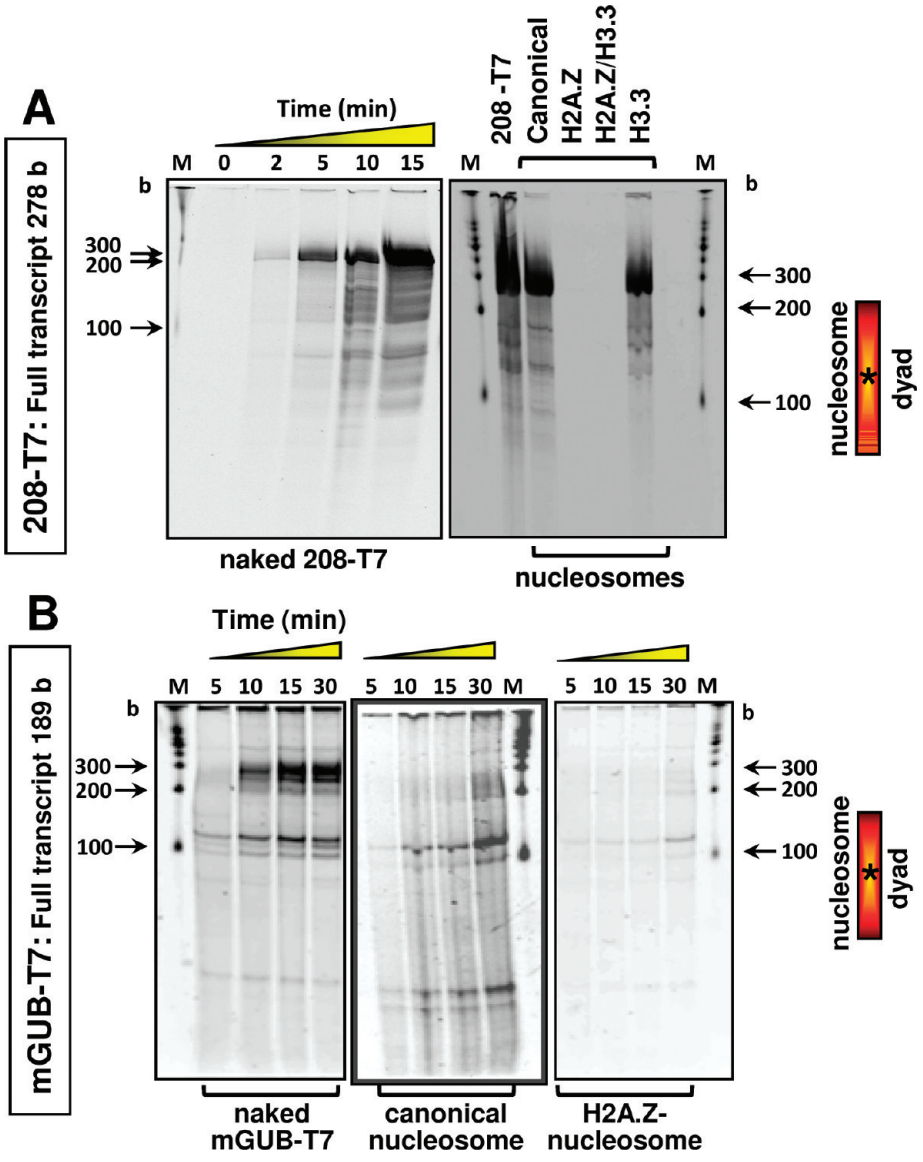

FIGURE 4: Analysis of RNA transcripts on 12% urea−polyacrylamide gels following DNase I treatment of the samples. The rectangles on the right depict the location of the nucleosome on the respective sequence, with the dyad axis marked with an asterisk. (A) Time course of naked 208-T7 transcription (left panel) and transcription of 208-T7 nucleosomes for 30 min (right panel). (B) Time course of transcription on mGUB-T7 naked DNA and reconstituted nucleosomes. Note the presence of several closely situated bands at and above the expected position of the fully denatured 189-bp runoff transcript. These bands may arise from residual stable secondary structures in the transcript. Indeed, analysis of the RNA sequence by RNAfold software (http://nar.oxfordjournals.org/cgi/content/full/gkn188v1) revealed a rather complex secondary structure. The three boxes are from the same gel, rearranged for clarity.

these papers. First, Mavrich et al. (*10*) presented genome-wide data showing that in ~2000 "paused" *Drosophila* genes (i.e., genes which are not transcribed but in which Pol II initiated transcription and then paused at the beginning of the transcribed region, ready to restart transcription upon activation signals) Pol II is actually stalled at a H2A.Z-containing nucleosome. Of note, there was no enrichment of Pol II at this position in a large set (close to 9000) of nonpaused genes or in ~800 genes whose +1 nucleosome does not contain H2A.Z. The authors state that "it is not known whether the +1 nucleosome is causative or just participatory in the pausing" (*10*). Our *in vitro* transcription data would suggest that the +1 (H2A.Z-containing) nucleosome is actually causative for the pausing. It must be noted that the first "pausing" H2A.Z-containing nucleosome is followed by a string of several well-positioned H2A.Z-containing nucleosomes (*10*). These may be viewed as a backup mechanism for pausing. Whatever mechanism removes the +1 nucleosome during transcription elongation must also remove the additional three to four H2A.Z-containing nucleosomes.

The second paper investigated changes in histone variant distribution that accompany activation of two inducible genes in human T cells (*40*). Induction of these genes was paralleled by depletion of H2A.Z, with concomitant deposition of H3.3 at a nucleosome immediately downstream from the transcription start site. In light of our results, the inhibitory H2A.Z-containing nucleosome must be removed for transcription to proceed.

Third, Felsenfeld and co-workers (*13*) find a light but consistent elevation of H2A.Z-only particles over the gene bodies and downstream from transcription start sites of silent genes. This observation may indicate that, at least in part, the lack of transcription in silent genes may be due to untranscribability of H2A.Z-containing nucleosomes.

Finally, Kumar and Wiggle (*41*) reported an interesting study on the involvement of H2A.Z-containing nucleosomes in

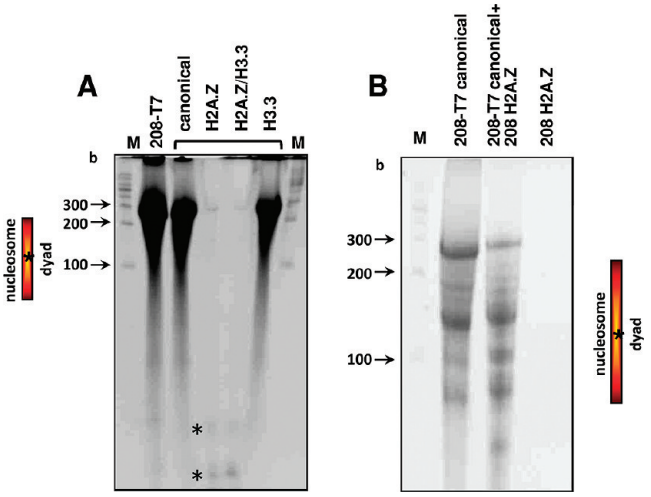

FIGURE 5: Does H2A.Z directly inhibit T7 RNAP activity? (A) Analysis of RNA transcripts on 208-T7 nucleosomes (30 min reaction) on 12% urea−polyacrylamide gels ran for shorter time; note the presence of very short transcripts marked as *, indicating that transcription of the sequence outside the confines of the particle occurs. (B) Analysis of transcription reactions containing 208-T7 canonical nucleosomes, an equimolar mixture of 208-T7 canonical nucleosomes and promoterless 208-H2A.Z particles, and promoterless 208-H2A.Z nucleosomes only. Promoterless H2A.Z-containing nucleosomes do not inhibit transcription on 208-T7 canonical nucleosomes.

mediating the response of *Arabidopsis* to elevated temperatures. They found a significant decrease (with temperature) of H2A.Z occupancy across all genes examined, independently of their transcriptional response. Restriction digestion accessibility studies on nucleosomes purified from wild-type and mutant plants (that are deficient in deposition of H2A.Z) suggested tighter wrapping of DNA around H2A.Z-containing histone octamers; this tighter wrapping is overcome by elevated temperatures. It is difficult to compare this study with our findings, since the *in vivo* effects could be mediated by posttranslational modification or by interactions with other factors.

What could be the molecular basis for the lack of transcription of H2A.Z-containing nucleosomes? The data on the biophysical stability of these particles are exceedingly controversial (discussed in detail in ref *4*; for newer data see refs *42* and *43*). Of note, in a model that predicts nucleosome positioning genome-wide in yeast (*44*), the most stable nucleosomes coincide with the H2A.Z nucleosomes located *in vivo*. We find that nucleosomes containing H2A.Z are slightly more compact than their canonical counterparts, as judged by their electrophoretic mobility and sedimentation behavior (*43*). We also find no significant differences in the stability of canonical, H2A.Z-containing, H3.3-containing, and H2A.Z/H3.3 hybrid reconstituted nucleosomes, as judged by their dissociation as a function of salt concentration (*43*). Moreover, the stability of all of these reconstituted particles is almost indistinguishable from that of native nucleosome core particles (*45*, *46*). We must note that in terms of stability of variant nucleosomal particles, our data differ from those of Jin and Felsenfeld (*12*). This difference may reflect the presence of histone postsynthetic modifications in the native particles of Jin and Felsenfeld (*12*). Our reconstituted particles contain recombinant histones devoid of any modifications; thus, their behavior (stability and transcribability) reflects the intrinsic properties of the variant particles, unaffected by modifications. In this regard, it is important to note that Ishibashi et al. (*42*) reported no effect of H2A.Z acetylation on the stability of the particle. Thus, the reconstituted H2A.Z/H3.3 particles do not differ in stability from the canonical particles but do differ in transcribability; thus, the two properties are not directly connected.

The activities of several histone-modifying enzymes and chromatin remodelers are negatively affected by H2A.Z, making H2A.Z-containing nucleosomes relatively immobile and refractory to remodeling (*34*). Clearly, though, H2A.Z-containing nucleosomes are transcribed *in vivo* by eukaryotic RNA polymerases (*9*, *10*, *40*). The downstream H2A.Z-containing nucleosome of the two surrounding transcription start sites (*9*) is evicted by the transcription process itself (*47*) through an unknown mechanism. Through their ability to remove H2A/H2B dimers during elongation, the chromatin-specific elongation complex FACT (*48*) or histone chaperone Nap1 (*2*) could be essential for transcribing H2A.Z-containing nucleosomes. Of note, H2A.Z interacts genetically with elongation factor TFIIS in yeast (*49*).

The present results make an essential point: H2A.Z-containing nucleosomes differ significantly in structure from their canonical counterparts, making them refractory to transcription with a model polymerase. In the future, we will directly compare the transcriptional behavior of these particles using either eukaryotic cell-free systems or purified eukaryotic transcription factors. We are now searching for eukaryotic protein partners of histone H2A.Z that may help to overcome the transcriptional block imposed by the presence of H2A.Z in nucleosomes.

Finally, we did not observe any effects of the presence of H3.3 on transcription. The behavior of "hybrid" particles containing both H2A.Z and H3.3 was entirely dominated by the presence of H2A.Z.

In the introduction, we posed the question of whether the presence of H2A.Z and H3.3 variants is just a reflection of high nucleosome turnover or whether H2A.Z and H3.3 endow certain structural characteristics to the nucleosomes that directly affect their behavior. The answer seems to be different for the two variants. Based on our *in vitro* results, nucleosomes are transparent to the presence of H3.3; thus, H3.3 may indeed be just a marker of chromatin regions in flux. The presence of H2A.Z, on the other hand, seems to have direct consequences for the transcriptional properties of the particles. The effects of H2A.Z are finely tuned by the underlying DNA sequence.

The overall results from our study could be partly rationalized in terms of the relative strengths of histone−DNA interactions in the nucleosome, as measured by single molecule DNA unzipping experiments (*50*). The point of contact between loop 1 of H2A and DNA is in a high interaction region. This is one of the regions where major differences exist between H2A and H2A.Z (Figure 1). Thus, we suggest that the substitution of H2A.Z for H2A may lead to strengthening of these contacts, creating a high barrier for RNA polymerase traversal. Additionally, the contacts between the two H2A.Z molecules in a particle [which are stronger than those between the two H2A molecules in canonical core particles (*27*)] may play a role in the transcriptional behavior of the H2A.Z-containing particles. On the other hand, the differences between H3 and H3.3 are located in a region (beginning of α-helix 2) which does not contact the DNA directly and thus

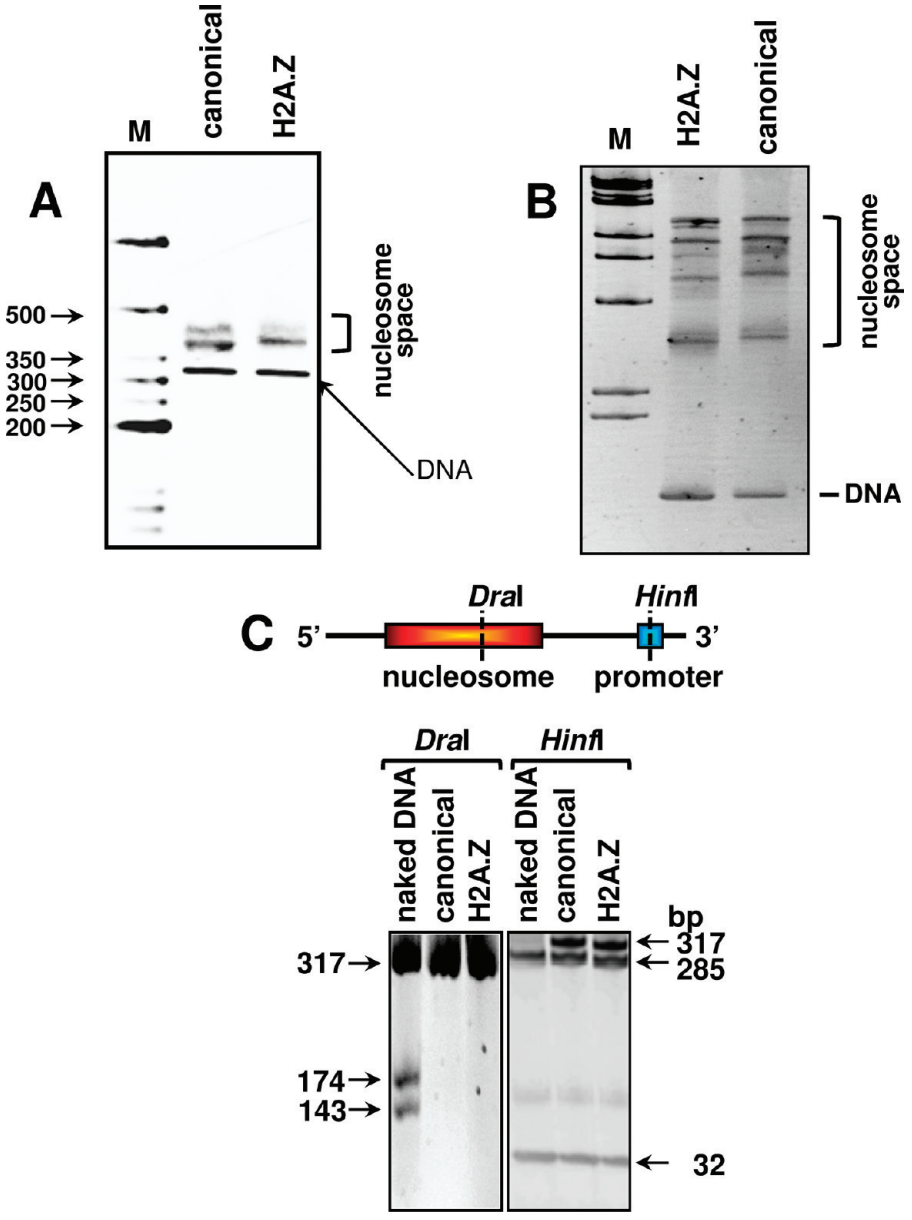

FIGURE 6: Analysis of canonical and variant nucleosomes reconstituted on the 208-T7 317-bp sequence. (A) Band-shift analysis on native agarose gels. Note that in both the canonical and H2A.Z nucleosome preparations the ratio between the two bands occupying the nucleosome space is the same: two-thirds of the DNA organized in nucleosomes contain one nucleosome (lower band) and one-third contains two nucleosomes. (B) Occupancy of alternative nucleosome positions in canonical and H2A.Z-containing nucleosomes, as analyzed by 5.5% native polyacrylamide electrophoretic gels. The positions of the naked DNA template and the reconstituted nucleosomes are marked. (C) Accessibility of the T7 RNAP promoter region to restriction nuclease digestion (restriction map above the agarose electrophoretic gel). Note that *Dra*I digested only about one-third of the naked DNA to produce the expected 174- and 143-bp fragments (changing the digestion conditions did not improve the activity of the enzyme at hand). What is important is that both the canonical and the H2A.Z reconstitutes showed no digestion, indicating that the *Dra*I site was protected equally well in both particles, as expected. *Hin*fI digested naked DNA to >90%, while in canonical and H2A.Z-containing nucleosomes, the level of digestion was practically indistinguishable (about 70% of the input DNA was digested in both cases, as quantified by scanning of the stained gels).

may not contribute to the overall strength of the particle. Hence, the H3.3-containing nucleosome is not expected to differ in its transcriptional behavior from the canonical H3-containing particle, which is what we observe.

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