RNA Polymerase-specific Nucleosome Disruption by Transcription in Vivo*

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The nucleosomal chromatin structure within genes is disrupted upon transcription by RNA polymerase II. To determine whether this disruption is caused by transcription per se as opposed to the RNA polymerase source, we engineered the yeast chromosomal HSP82 gene to be exclusively transcribed by bacteriophage T7 RNA polymerase in vivo. Interestingly, we found that a fraction of the T7-generated transcripts were 3’ end processed and polyadenylated at or near the 3’ ends of the hsp82 and the immediately downstream CIN2 genes. Surprisingly, the nucleosomal structure of the T7-transcribed hsp82 gene remained intact, in marked contrast to the disrupted structure generated by much weaker, basal level transcription of the wild type gene by RNA polymerase II under non-heat shock conditions. Therefore, disruption of chromatin structure by transcription is dependent on the RNA polymerase source. We propose that the observed RNA polymerase dependence for transcription-induced nucleosome disruption may be related either to the differential recruitment of chromatin remodeling complexes, the rates of histone octamer translocation and nucleosome reformation during polymerase traversal, and/or the degree of transient torsional stress generated by the elongating polymerase.

Eukaryotic genomes are packaged in vivo into chromatin, whose basic repeating unit, the nucleosome, consists of two copies of each of histones H2A, H2B, H3, and H4 along with core and linker DNA sequences of about 200 bp1 (reviewed in Ref. 1). It has long been recognized that the chromatin structure of transcriptionally active class II genes exhibits an increase in DNase I sensitivity and a disrupted nucleosomal structure (reviewed in Refs. 1 and 2). Even relatively weak transcription by RNA polymerase II leads to a perturbation in nucleosomal structure (3).

In vitro transcription experiments with nucleosomal templates reveal that the core RNA polymerase II cannot traverse through nucleosomes (4), suggesting that in vivo the association between the core enzyme and accessory proteins may be important for template traversal. Indeed, the RNA polymerase II transcription complex can be associated with many accessory proteins, which include elongation factors (reviewed in Ref. 5), SWI/SNF chromatin remodeling factors (6), and RNA capping (reviewed in Ref. 7), splicing (reviewed in Refs. 8 and 9), cleavage, and polyadenylation activities (10). Several of these activities appear to be targeted through hyperphosphorylation of the carboxyl-terminal domain of RNA polymerase II (10–12). In vivo these complexes may be so large that during transcriptional elongation, instead of moving along a chromatin template, the template itself may be reeled through fixed nuclear structures (13, 14). Indeed, the hyperphosphorylated form of elongating RNA polymerase II is quantitatively recovered in the nuclear matrix fraction (15). On the other hand, the results of in vitro experiments reveal that single subunit prokaryotic RNA polymerases such as bacteriophage T7 can readily transcribe through nucleosome arrays (16–21). Interestingly, however, recently a protein complex termed FACT (facilitates chromatin transcription) has been purified that allows the stalled RNA polymerase II molecules to enter into productive transcription through short chromatin templates in vitro (22). In addition, in vitro transcription through nucleosomal templates by RNA polymerase II can also be stimulated under certain conditions by the SWI/SNF chromatin remodeling complex (23). Finally, because the activator domains of transcription factors associated with class II genes sometimes are involved in recruiting histone acetylase complexes (24, 25), it is conceivable that local histone acetylation may facilitate RNA polymerase II traversal through nucleosomes in vivo.

Insight into nucleosomal structural alterations during traversal by the SP6 prokaryotic RNA polymerase and by eukaryotic RNA polymerase III has come from model in vitro transcription experiments of Felsenfeld and co-workers (26–29). These polymerases pause near the nucleosome dyad axis, creating intranucleosomal DNA loops, to which histone octamer translocations may occur. In other words, the histones never leave the DNA template but are internally transferred to upstream positions.

To determine if there is modification of the chromatin template in vivo upon transcriptional elongation that is specific to the RNA polymerase employed, we have compared the chromatin structures generated by transcription of the same sequence in Saccharomyces cerevisiae by two different RNA polymerases. We report here that high level transcription by the T7 RNA polymerase has no detectable effect on modifying chromatin structure, whereas even very weak transcription of the same sequence by RNA polymerase II leads to a marked disruption in nucleosomal structure. These results suggest that either proteins associated with the RNA polymerase II transcription machine, the rates of histone octamer translocation and nucleosome reformation, and/or transient torsional stress modu-
late chromatin structure during template traversal in vivo and that chromatin structural changes are not simply the consequences of transcription per se.

**EXPERIMENTAL PROCEDURES**

*Strains, Plasmids, and Growth Conditions—S. cerevisiae Y303-1B (MATa ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100) served as the parent strain for construction of isogenic mutant strains. The HSP82 promoter was replaced by the bacteriophage T7 promoter and mutations in the TATA box and HSE1 (T2P2, Ref. 30) to obtain the T7hsp82 strain. The T7hsp82 strain was further manipulated by placing a T7 terminator dimer within the coding region of HSP82 gene to generate T7hsp82+pTD (see below and Fig. 1 for details). These strains were transformed either with the 2-μm yeast plasmid YEp401NLEU* or designated GAL-T7, or the integrating construct Ylp401NLEU* as indicated in the figure legends (Ref. 31; kindly provided by Christopher Greer, University of California, Irvine), which carry the T7 polymerase gene under control of the inducible GAL1 promoter. The empty plasmid (URA3) without the T7 polymerase gene served as a control. The plasmid Ylp5 (New England Biolabs) carrying the URA3 marker was used for HSP82 gene integration and gene replacement. Wild-type cells were grown in yeast extract/peptone medium (YPE) containing 2% glucose (32). Yeast transformations were done by the lithium acetate method (33, 34). The strains T7hsp82 and T7hsp82TD carrying either an empty or GAL-T7 plasmid or integral were grown in selective medium lacking leucine or uracil (32). All the strains were grown at 30 °C to A<sub>600</sub> = 0.8 (wild type) or 1.2 (T7hsp82–TD). From 10 ml overnight stationary cultures grown in selective medium containing 2% glucose, 150 μg/ml ampicillin, and 100 μg/ml kanamycin, a 15 μl aliquot was used to inoculate 1 liter of selective medium containing 2% raffinose, 3% galactose and grown in 2-liter flasks with shaking at 250 rpm for 15 h.

Substituting the T7 Promoter for the HSP82 Promoter—PCR-splicing by overlap extension (35) followed by two-step gene replacement was used to place the T7 bacteriophage promoter (~18 to +5) (36) and the T2P2 mutations (30) in place of wild type promoter of the HSP82 gene. The nucleotide sequences (5' to 3') of forward and reverse primer pairs used in two independent PCR reactions with a plasmid template bearing the T2P2 mutations (30) for splicing by overlap extension purposes were as follows: GGAATAAAGCTTAATCGG (~281 to +263 of HSP82) and CCCCCTATAGCTGTTATAGCGGAAGAAAATGACC (~31 to +17 lower strand T7 promoter sequences fused to ~18 to ~34 HSP82 sequences); CGACTCCTATAGGAGACCTGATAGAAAAATAGGCCTC (~12 to +5 upper strand T7 promoter sequences fused to +6 to +26 sequences of HSP82) and CCAAACTTTTTTCTGTCCTGG (~ +265 to +284 sequences of HSP82). The T2P2T7hsp82 PCR fragment (~281 to +284) was first cloned into the yeast integration vector Ylp5. Then it was cleaved at a unique BglII site (~289) and transformed into the wild type strain for homologous recombination. After initial selection for URA3 expression, those cells that subsequently had lost URA3 by undergoing recombination between duplicated adjacent sequences were isolated by the counter selection technique (37). Retention of the T2P2T7 HSP82 PCR fragment at its genetic locus was confirmed by Southern analysis.

Placing a T7 Terminator Dimer Near the 3' End of the T7hsp82 Gene—A 344-bp BamHI/HindIII fragment containing a dimer of the BglII fragments of pET-3 (36), the T7 RNA polymerase terminator, was excised from plasmid pTT2t (obtained from Alison Bertuch of the University of Rochester) and ligated into the EcoRI site (~1601) of the HSP82 gene on YlpHSP82 plasmid (carries a 2.89-kb BglII-ClaI fragment of the HSP82/CIN2 genes) using BamHI/EcoRI and HindIII/EcoRI adapters. This plasmid was linearized at MluI site and transformed to the T7hsp82 strain. The initial selection for URA3 expression and counter-selection were done as described earlier (37). Retention of the terminator dimer in the forward orientation at the EcoRI site (~1601) was confirmed by diagnostic PCR and restriction analysis.

**Northern Analysis**—RNA was isolated by one of two procedures as described elsewhere (3, 38). Northern analysis was performed as reported previously (3). Total RNA samples (8 μg) or poly(A) RNA, isolated using a Stratagene kit (poly(A) Quick mRNA Purification Kit number 200349) were separated in triplicate (except for poly(A) RNA) on the same 1.25% agarose/formaldehyde gels. After completing the run, formaldehyde was removed from the gels by 4 × 10 min washes at 65 °C in distilled H₂O. RNA was then transferred by capillary action to Zeta-Probe GT blotting membranes (Bio-Rad) with 2× TAE as transfer buffer. Following transfer, membranes were cut into three sections, rinsed in 2× TAE for 5 min, and allowed to air-dry. Membranes were prehybridized (65 °C for probe 1 and actin; 42 °C for probe 2) in Church-Gilbert buffer (250 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, and 7% SDS) and then hybridized separately to [32P]dCTP-labeled DNA probes (Amersham Pharmacia Biotech Oligolabeling Kit) in the same buffer and at the same temperature as above. Probe 1 was 753-bp XmnI-EcoRI fragment from +848 to +1601 of the HSP82 gene, and probe 2 was a 100-mer from +2190 to 2290 of the same gene (see Fig. 1). To normalize the RNA loading, one filter was hybridized with an actin probe isolated from pGEMACTIN (40), and the +318 to +1063 coding sequence was PCR-amplified and labeled as mentioned above. Following hybridization, membranes were washed 3× in 0.2× SSC, 2% SDS plus 20 μM sodium phosphate buffer, pH 7.0, for 15 min at 53 °C (probe 2) or 65 °C (probe 1 and actin), and blots were exposed to PhosphoImaging screens at room temperature or to x-ray film with intensifying screens at ~70 °C. Phosphor screens were scanned with a Molecular Dynamics PhosphorImager, and the signals were quantitated using ImageQuant software or imaged using Adobe Photoshop 3.0.

**DNase I Chromatin Footprinting Assay**—Yeast cells were converted to spheroplasts using oxaIyticase (Enzonogenetics) in the presence of 20 mM sodium azide, and nuclei were isolated and digested with DNase I as described previously (3). DNA was prepared as described (41). For naked DNA controls, DNA was isolated from nuclei or spheroplasts prior to DNase I treatment. EcoRI-digested DNA samples (10 μg) were separated by electrophoresis on 2% agarose gels in 1× TPE (90 mM Tris phosphate, 2 mM EDTA) and transferred to the Zeta-Probe GT membrane (Bio-Rad) by the capillary method in 0.4× NaOH, 0.2× SSC. Following transfer, membranes were neutralized with 40 mM sodium phosphate buffer, pH 7.0, for 10 min and hybridized overnight at 65 °C with a [32P]labeled probe 694-bp EcoRI-EcoRI fragment from +911 to +1601 of the HSP82 gene (described above) for indirect end labeling (42). After hybridization, filters were washed 3× for 20 min at 65 °C with 0.1× SSC, 2% SDS plus 20 μM sodium phosphate buffer, pH 7.0. Signals were then visualized as described above.

**RESULTS**

Engineering T7 RNA Polymerase-mediated Transcription of the Yeast Chromosomal HSP82 Gene—Fig. 1 illustrates our experimental strategy to address whether disruption of nucleosome structure caused by transcription in vivo exhibits any specificity for the RNA polymerase source. We inactivated the yeast HSP82 gene promoter for transcription by RNA polymerase II by creating base changes in heat shock element 1 (designated HSE1) and the TATA box (30). By using PCR...
techniques, we substituted 23 bp of the HSP82 gene sequences using a downstream probe (data not shown). Although these results contrast with previous reports in which class II gene transcripts generated in vitro cleavage and polyadenylation can occur post-transcriptionally in cell-free extracts (44, 45) or in purified systems free of RNA polymerase II (46, 47), we investigated the possibility that such events may also occur in vivo for these T7-generated transcripts. We affinity fractionated total RNA on oligo(dT) and found 2.3- and 3.3-kb-long T7hsp82 transcripts that were indeed poly(A)⁺ (Fig. 3, lane 4). Furthermore, it is particularly striking that RNA species >2.3 kb but <3.3 kb seen as a smear in the input did not fractionate with poly(A)⁺ material (Fig. 3, compare lanes 3 and 4). Apparently, the processing signals at the ends of the hsp82 and immediately downstream CIN2 genes are used independently of RNA polymerase II transcription. Furthermore, we verified that the longer sequences possessed both hsp82 and CIN2 gene sequences using a downstream probe (data not shown). Although these results contrast with previous reports in which class II gene transcripts generated by either RNA polymerase I or III were found not to be 3’-polyadenylated in mammalian cells (48, 49), our estimates suggest that such post-transcriptional processing in yeast is not very efficient and may only represent a few percent of the total transcript population (data not shown); furthermore, we can not rule out a trans, post-transcriptional role for RNA polymerase II in such processing as has been demonstrated recently by in vitro experiments (50).

Chromatin Structural Analyses—To investigate transcription-induced changes in the chromatin structure of hsp82 alleles, we used the indirect end-labeling technique to map nucleosome cutting sites at low resolution (±20 bp) toward the 3’ end of the hsp82 gene as well as further downstream into the CIN2 gene and beyond (42). Previous studies have shown that in the case of both basal and heat shock-induced transcription exhibited by the wild type gene, the chromatin structure within the

FIG. 2. Northern analysis shows that engineered hsp82 alleles are actively and faithfully transcribed exclusively by T7 RNA polymerase. Total RNA was isolated from non-heat-shocked wild type (WT) cells grown in medium with glucose as the carbon source or from strains bearing T7hsp82 as the terminator dimer (Term) alleles grown in medium with galactose as the carbon source and either harboring an empty expression vector or GAL/T7 as indicated. A, Northern filter hybridized with hsp82 gene-specific probe 2 (see Fig. 1A for map position); B, probe 1 (see Fig. 1A for map position); C, ACT1 probe to detect actin gene transcripts as a loading control.

FIG. 3. Oligo(dT) chromatography reveals that a fraction of T7 RNA polymerase-generated hsp82 transcripts are processed and polyadenylated. Total RNA was isolated from heat-shocked wild type (WT) cells grown in medium with glucose as the carbon source or from T7hsp82 cells grown in medium with galactose as the carbon source, harboring a GAL/T7 expression vector integrated at the LEU2 locus. Northern filter of total RNA (T) or the poly(A)⁺ fraction (A⁺) hybridized with hsp82 gene-specific probe 2 (see Fig. 1A for map position).
transcription unit exhibits a half-nucleosomal DNase I cleavage periodicity of about 80-bp increments, which we have termed a "split nucleosomal structure" (3). However, only a whole nucleosome DNase I cleavage periodicity is exhibited in the same region in genes possessing promoter mutations that abolish basal transcription (3).

As shown in Fig. 4B for two independent experiments (Exp. 1, lanes 2–5), either in the absence or presence of T7 RNA polymerase-mediated high level transcription of T7hsp82, three translationally positioned nucleosomes at the 3' end of the hsp82 gene can be detected, which are bounded by two whole nucleosome-spaced DNase I cutting sites and a hypersensitive site at the gene end (open circles and arrows). However, in agreement with our previous studies (3, 51), in wild type non-heat-shocked yeast the chromatin structure in the corresponding region is disrupted and exhibits a half-nucleosomal DNase I cleavage periodicity (Fig. 4A, lane 1, closed circles). We also investigated the chromatin structure in T7hsp82TD and found that, either in the absence or presence of T7 RNA polymerase-mediated high level transcription, no detectable disruption of nucleosomal structure in the relevant regions could be detected in two independent experiments (Fig. 4C, Exp. 1, lanes 6 and 7, and Exp. 2, lanes 8 and 9). Introduction of the terminator dimer sequence, however, leads to the irregular positioning of one nucleosome immediately downstream (Fig. 4C, lanes 6–9, overlapping open circles).

Nevertheless, we conclude that chromatin structural alterations exhibit an unexpected dependence on the RNA polymerase used for transcription.2

2 In rich medium (YPGal), we obtained identical results for the T7hsp82TD strain expressing an integrated copy of the T7 RNA polymerase gene. However, in YPD medium the T7hsp82 strain exhibits slight splitting of one nucleosome in the absence of T7 RNA polymerase expression, which we attributed to leaky RNA polymerase II transcription based on Northern analyses. In the same strain such splitting is mildly enhanced in YPGal medium upon expression of the integrated copy of T7 RNA polymerase, which we attributed to increased leaky RNA polymerase II transcription triggered by a stress response.
The yeast chromosomal hsp82 gene offers a unique opportunity to examine with high sensitivity nucleosomal structural changes in response to traversal by RNA polymerases. Previous studies have established that transcriptionally active and rapidly switching their transcription on and off has not been possible.

We have observed that transcription by RNA polymerase II, but not by T7 RNA polymerase, disrupts the chromatin structure within the chromosomal yeast hsp82 gene. Thus, the act of transcription per se does not lead to nucleosome alterations, and there appears to be something special about the RNA polymerase II complex. We discuss below possible mechanisms that may account for these observations.

Proteins associated with the RNA polymerase II elongation complex, such as elongation factors (5), RNA processing components (8–12), chromatin remodeling machines (6, 23, 52), or FACT (22), may be responsible for generating disrupted nucleosomal structures. In this regard it is interesting that chromatin remodeling complexes have been recently shown to introduce reversible but persistent changes in nucleosome conformation without displacing the histones that lead to heightened DNase I sensitivity in vitro during the association with the chromatin remodeling complexes (53–55). In addition, because transcriptionally active class II genes appear to bear nucleosomes containing highly acetylated histones (24, 25), it is conceivable that local histone acetylation may be connected in part to nucleosomal structural changes mediated through RNA polymerase II targeting mechanisms.

The differential chromatin structures generated by the RNA polymerase source may be related to the rates of nucleosome repair or reformation after histone octamer translocation during RNA polymerase traversal (26–29). On naked DNA substrates the T7 RNA polymerase elongation rate is much faster than that by RNA polymerase II, which is probably also the case on nucleosomal substrates (see Introduction). Previous studies employing in vitro transcription of a nucleosomal substrate with RNA polymerase III, an enzyme of approximately the same size as RNA polymerase II, have revealed marked pausing (29). Similar pausing by RNA polymerase II may retard the kinetics of nucleosome reformation and lead to the detection of nucleosome transfer intermediates scored here as split nucleosomes. By contrast, the more rapid traversal by the T7 enzyme may allow more time between successive transcription cycles to allow for nucleosome repair.

Another possibility, which is not mutually exclusive of the above models, is that transcription by RNA polymerase II may generate far more transient torsional stress in the chromatin DNA template as compared with that generated by T7 RNA polymerase, and this stress may be responsible for disrupting nucleosomal structure even at a distance. As described in the Introduction, elongating RNA polymerase II is associated with many accessory proteins and may be anchored to insoluble nuclear structures. In this case during transcription the chromatin would be reeled through, and every 10-bp step of traversal would generate one positive supercoil downstream and one negative supercoil upstream of the anchored polymerase complex, whose relaxation by the DNA topoisomerases may kinetically lag (2, 56). Furthermore, it is known in S. cerevisiae that nuclear anchoring of circular templates outside of transcription units greatly increases localized torsional stress, presumably introduced by nearby RNA polymerase II transcription (57, 58). By contrast, the smaller single subunit T7 RNA polymerase may rotate nearly freely around the template during traversal because of low hydrodynamic drag, thereby not generating substantial twin domains of DNA supercoiling, as has been shown in Escherichia coli for transcription-translation complexes encoding non-membrane-bound proteins (59). We have previously demonstrated that positive supercoiling disrupts nucleosomal structure and generates DNase I sensitivity (41). Current modeling of nucleosomal alterations caused by positive supercoiling features H3–H4 tetramers wrapping a right-handed superhelix with the displacement of H2A–H2B dimers (60, 61).

In conclusion, we have detected an unexpected polymerase-specific disruption in nucleosomal structure, and we have discussed possible mechanisms to account for differential structural alterations, some of which are experimentally testable. A crucial unanswered question, however, remains. Is the observed disruption in nucleosome structure a requirement for RNA polymerase II traversal or is it simply a by-product of the process?

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