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Histone octamer dissociation is not required for transcript elongation through arrays of nucleosome cores by phage T7 RNA polymerase *in vitro*

(RNA synthesis/5S RNA-encoding DNA/chromatin/dimethyl suberimidate)

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ABSTRACT **We have examined whether dissociation of the histone octamer is required for elongation of RNA transcripts through arrays of nucleosome cores *in vitro*. Control or dimethyl suberimidate-crosslinked histone octamers were reconstituted onto supercoiled, closed circular pT207-18 DNA, which contains tandemly repeated 207-base-pair (bp) 5S rDNA nucleosome positioning sequences inserted between the T7 and SP6 transcription promoters of pGEM-3Z. Double label transcription experiments showed that there was little or no effect of extensive crosslinking of the histone octamers on transcription initiation and elongation by T7 RNA polymerase *in vitro*. Continuous regularly spaced linear arrays of either crosslinked or control nucleosome cores were obtained by digesting reconstituted nucleosomal pT207-18 templates with *Dra* I, a site that is protected from digestion by the presence of positioned nucleosome cores in the 207-bp sequence. After *in vitro* transcription with T7 RNA polymerase, an RNA ladder with 207-nucleotide spacing was obtained from templates reconstituted both with crosslinked and with control histone octamers, demonstrating clearly that neither partial nor complete dissociation of the histone octamer is essential for transcription elongation through arrays of nucleosome cores *in vitro*.**

The mechanism of passage of RNA polymerase through nucleosomes has been the subject of intense interest over the past few years. It has been shown clearly in *in vitro* experiments that bacteriophage SP6 polymerase can transcribe through one nucleosome (1, 2) or short stretches of a few nucleosomes (3). Recent work from this laboratory has shown that T7 RNA polymerase can transcribe through regularly spaced arrays of at least 10 positioned nucleosome cores, but the efficiency of transcription elongation is significantly decreased compared to free DNA templates (4). Similar results have been obtained in studies of T7 RNA polymerase transcription through linear DNA fragments containing 4–7 nucleosome cores (5). Greater inhibition of transcription elongation is observed in transcription of nucleosomal templates by RNA polymerase II (6).

Hence, abundant evidence indicates that although RNA polymerases can transcribe through individual nucleosome cores, arrays of nucleosome cores do present a significant obstacle to transcription elongation. At this time how RNA polymerase gains access to DNA wrapped into nucleosomes during transcription elongation remains unclear. A number of models have been proposed for this process that involve the transient disruption of nucleosome structure during the process of transcription elongation via displacement of histones from DNA or via splitting or unfolding of the nucleosome (reviewed in ref. 7). Proposed mechanisms involve the opening of the nucleosome into halves, each containing a heterotypic tetramer (8); the formation of an unfolded lexosome structure containing nonhistone proteins (9); the splitting of nucleosomes by transcription-induced positive supercoiling (10); the loss of an (H2A-H2B) dimer, resulting in a depleted nucleosome that more easily complexes with RNA polymerase (11); the progressive transient displacement of histone H2A-H2B dimers as RNA polymerase passes through the nucleosome (12); and the transfer of histone octamers to sites behind the transcribing RNA polymerase (13, 14). The physiological validity of these mechanisms remains speculative for lack of definitive experimental evidence.

In a previous study (4) we made a 6.6-kilobase-pair (kbp) DNA construct, pT207-18, which contains an insert of 18 tandem repeats of the 207-bp nucleosome positioning sequence from the *Lytechinus variegatus* 5S RNA gene adjacent to a bacteriophage T7 transcription promoter. Because nucleosome cores are assembled into regularly spaced positioned arrays on these tandem repeat sequences (15–18), the pT207-18 construct can be used to assay the efficiency of transcript elongation through homogeneous arrays of regularly spaced nucleosome cores in a well-defined *in vitro* system. This construct was used to show that bacteriophage T7 RNA polymerase can elongate transcripts through arrays of up to at least 10 nucleosome cores, but that with increasing numbers of nucleosome cores transcription elongation is partially inhibited. In the current study, we use the pT207-18 construct to assay the effects of extensive chemical crosslinking of histone octamers on elongation of transcripts through reconstituted arrays of nucleosome cores by T7 RNA polymerase *in vitro*. These experiments directly test models for transcription elongation through nucleosome cores that involve splitting or dissociation of the octamer prior to or during transcription. We demonstrate that dissociation of histone octamers is not necessary for transcription elongation through nucleosome cores *in vitro*. It is shown that nucleosomal arrays reconstituted from histone octamers that have been extensively crosslinked with dimethyl suberimidate behave similarly to arrays reconstituted from control histone octamers in transcription assays with T7 RNA polymerase. Transcription occurs through arrays of both control and crosslinked nucleosome cores, albeit at a lower efficiency than for free DNA templates. Thus, dissociation or splitting of the histone octamer is not essential for transcription elongation through nucleosome cores *in vitro*.

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

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MATERIALS AND METHODS

Preparation of Histone Octamers. Nucleosome core particles and histone octamers were prepared from HeLa nuclei as described (4). Crosslinked histone octamers were prepared essentially as described by Stein *et al.* (19). Nucleosome core particles at 100 μg/ml were crosslinked in 100 mM sodium borate (pH 10) containing dimethyl suberimidate (Pierce) at 10 mg/ml for 40 min at 25°C. This material was then dialyzed against 100 mM sodium borate (pH 10) at 4°C, concentrated, and crosslinked a second time with dimethyl suberimidate as described above. The crosslinked particles were then dialyzed against 10 mM Tris·HCl, pH 6.85/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride (PMSF) and refractionated on 5–20% sucrose gradients. The purified particles were then extracted with 3 M NaCl/10 mM Tris·HCl, pH 7.4, several times to precipitate histones and to remove DNA before storage in 10 mM Tris·HCl, pH 7.4/0.5 mM EDTA/0.1 mM PMSF. The purity and integrity of the individual histones and the extent of histone crosslinking were determined by 18% (wt/vol) polyacrylamide gel electrophoresis (20), followed by staining with Coomassie brilliant blue R.

Nucleosome Core Reconstitution. Nucleosome cores were reconstituted onto supercoiled plasmid DNA by using a modification of the salt dilution method of Germond *et al.* (21). In reconstitutions with control histone octamers, octamers were mixed with 12.5 μg of supercoiled pT207-18 DNA in 2 M NaCl/10 mM Tris·HCl, pH 7.4/10 mM sodium butyrate/0.5 mM EDTA/0.1 mM PMSF in an initial volume of 20 μl. Samples were diluted at successive intervals of 30–60 min to contain 1.12 M, 0.8 M, 0.6 M, 0.4 M, and finally 0.12 M NaCl in the same buffer at room temperature (24°C). Crosslinked octamers were initially mixed in 1 M NaCl and diluted in steps to 0.8 M, 0.6 M, etc., as described above. Samples were centrifuged at 12,000 × *g* for 10 min to remove aggregated material and stored on ice until use. All assays were performed within 1 week of sample preparation. For analysis of DNA topology, reaction mixtures containing reconstituted chromatin volumes corresponding to 6 μg of input plasmid DNA, 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, and 120 units of chicken erythrocyte topoisomerase I were incubated for 1 hr at 37°C. Next, SDS and EDTA were added to 0.2% and 15 mM, respectively, followed by phenol/chloroform extraction and ethanol precipitation. The DNA topology of the relaxed chromatin samples was analyzed by electrophoresis in agarose gels containing chloroquine diphosphate as described (4).

Transcription Reactions. Transcription assays were performed in 25-μl reaction mixtures containing 225 ng of DNA or chromatin (final A_{260} of 0.18); 40 mM Tris·HCl (pH 7.5); 6 mM $MgCl_2$; 0.2 mM EDTA; 2 mM spermidine; 50 mM NaCl, 0.5 mM each of ATP, CTP, GTP, and UTP; 1 unit of RNasin (Promega) per μl; 3 units of chicken erythrocyte topoisomerase I; 10 μCi (370 kBq) of [γ-^{32}P]GTP; and 1 μCi of [^{3}H]UTP (final concentrations). Reactions were preincubated at 37°C for 15 min to relax chromatin templates, which was followed by addition of 200 units of T7 RNA polymerase (New England Biolabs) and subsequent incubation for an additional 20 min. Reactions were terminated by addition of EDTA to 25 mM on ice and analyzed by DE-81 filter binding assays (22). Transcription reactions were done in triplicate, and measurements were averaged.

In experiments involving restriction enzyme digestion of reconstituted chromatin with *Dra* I prior to transcription, 1.5 μg of reconstituted pT207-18 DNA was incubated with 50 units of *Dra* I restriction enzyme in 10 mM Tris·HCl, pH 7.5/50 mM NaCl/6 mM $MgCl_2$/2 mM EDTA/0.1 mM PMSF for 90 min at 37°C. Single-label transcription reactions were performed with 150 ng of *Dra* I-digested pT207-18 template in the presence of 5 μCi of [α-^{32}P]GTP under conditions identical to those used in double-label experiments. Transcription reactions were analyzed by DE-81 filter binding assays and electrophoresis in 6% polyacrylamide gels containing 8.3 M urea. Appropriate sample volumes were loaded onto gels for comparison on the basis of equal ^{32}P incorporation.

RESULTS

Crosslinking of Octamers and Nucleosome Core Reconstitution. Stein *et al.* (19) demonstrated that dimethyl suberimidate-crosslinked histone octamers can be used to reconstitute nucleosome core particles that have properties quite similar to those of native nucleosome cores as assessed by circular dichroism spectra, DNase I digestion, electron microscopy, and DNA supercoiling assays. Nucleosome core particles assembled from crosslinked histone octamers were only slightly more stable to thermal denaturation than native core particles in these studies. A modification of their procedure was used to produce extensively crosslinked histone octamers from HeLa nuclei. Crosslinked and control histone octamers were analyzed by SDS/PAGE as shown in Fig. 1. The crosslinked histone octamer migrates with an apparent molecular weight of ≈100,000 daltons, with no detectable contamination of lower molecular weight components.

In the current study, nucleosome cores were reconstituted onto supercoiled closed circular pT207-18 DNA from donor crosslinked or control histone octamers by salt dilution. Micrococcal nuclease digestion analysis of pT207-18 DNA reconstituted with either crosslinked or control histone octamers gave similar DNA ladders (not shown), in agreement with Stein *et al.* (19). The extent of reconstitution of nucleosome cores from crosslinked or control histone octamers onto the closed circular DNA templates was obtained from the levels of DNA supercoiling constrained by the nucleosome cores after relaxation with topoisomerase I. Based on a DNA linking number of −1 per nucleosome core (15, 23–25), change in the DNA linking number is taken as a

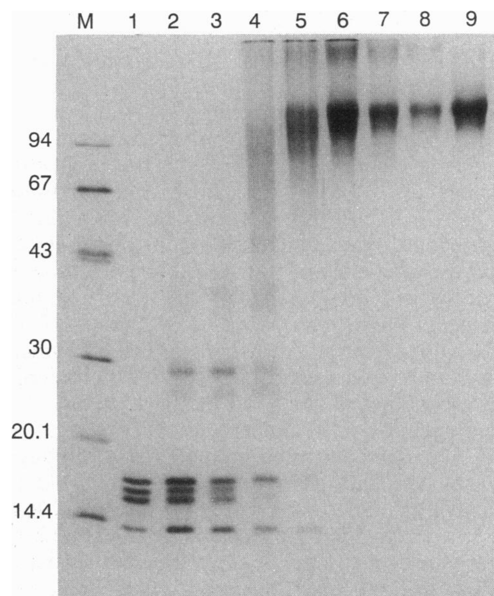

FIG. 1. SDS/PAGE analysis of histone octamers used for reconstitution. Lanes: M, molecular mass markers indicated in kDa; 1, control HeLa histone octamers; 2–7, analysis of histones from core particles crosslinked at pH 10 with dimethyl suberimidate at 10 mg/ml for 1, 2, 5, 10, 20, and 40 min; 8, histones from core particles crosslinked once for 40 min as above, followed by dialysis against 10 mM sodium borate, and a second time for 40 min with dimethyl suberimidate at 10 mg/ml; 9, histone octamers purified from twice-crosslinked core particles.

measure of the number of nucleosome cores assembled onto the closed circular DNA templates from control and crosslinked histone octamers.

Effects of Crosslinked Histone Octamers on Transcription. Previously, we have used the unique properties of T7 RNA polymerase to determine the effects of nucleosome cores on the initiation and elongation of transcripts from reconstituted closed circular DNA templates (4). Transcript initiation begins with the specific incorporation of a GTP residue, resulting in a γ-phosphate at its 5′ end (26). Hence, *in vitro* transcription in the presence of [γ-^{32}P]GTP will result in transcripts with the γ-^{32}P label incorporated only at their 5′ end and only once per transcript. This property can be exploited in double-label transcription assays that include both [γ-^{32}P]GTP and [^{3}H]UTP, which will be incorporated throughout the length of the transcript. The level of incorporation of the ^{32}P label into transcripts is proportional to the number of transcripts synthesized in a given reaction, whereas the incorporation of the ^{3}H label is proportional to the total number of nucleotides incorporated into transcripts. The amount of ^{3}H incorporated divided by the amount of ^{32}P incorporated is proportional to the average length of the transcripts produced in a given reaction. Using this assay, we have examined the separate effects of nucleosome cores on transcription initiation and elongation by bacteriophage T7 RNA polymerase.

The results of these transcription assays are shown in Fig. 2. In Fig. 2 *Top* the total nucleotide incorporation ([^{3}H]UTP incorporation) is given versus DNA linking number change, which measures the numbers of nucleosome cores reconstituted onto the pT207-18 template. Total nucleotide incorporation decreases sharply from templates reconstituted with either crosslinked or control histone octamers. The data show that crosslinking of histone octamers has little effect on the total incorporation of nucleotides into transcripts from reconstituted templates. In Fig. 2 *Middle*, the incorporation of ^{32}P into transcripts, which is proportional to the number of transcripts synthesized, is shown plotted against the DNA linking number change resulting from reconstitution. At lower levels of reconstitution, the relative number of transcripts produced from reconstituted templates is quite similar to that obtained from free DNA. However, with increased nucleosome core loading, a sharp drop in the relative numbers of transcripts produced is observed. Again, the inhibition pattern is similar for both templates reconstituted with control and crosslinked histone octamers. Fig. 2 *Bottom* shows that increased loading of nucleosome cores onto the DNA templates also results in a reduction in the length of the transcripts produced from these templates. Crosslinked and control nucleosomal templates behave almost identically in these assays, indicating that the effects of crosslinked histone octamers on transcript elongation are quite similar to those of control octamers. The behavior of the control reconstituted templates in these transcription assays is consistent with that observed in our previous studies (4).

Transcription Occurs Through Arrays of Crosslinked Nucleosome Cores. We have previously used the unique *Dra* I restriction site, which lies within the nucleosome positioning region of the 207-bp repeat sequences, to demonstrate that T7 RNA polymerase can transcribe through arrays of nucleosome cores. Reconstituted closed circular DNA templates are first digested with *Dra* I and are then used as transcription templates in reactions containing a single radiolabeled nucleotide, [α-^{32}P]GTP. Templates are truncated by cleavage at unprotected *Dra* I cleavage sites. Since a large excess of *Dra* I was used to digest the minichromosomes, only *Dra* I sites protected by the presence of nucleosome cores will not be cleaved. Transcripts produced from free DNA templates will extend only from the transcription start site to the *Dra* I site in the first adjacent 207-bp repeat. Transcripts longer than this length can only be produced by transcription through arrays of nucleosome cores. The length of the transcripts will correspond directly to the number of arrayed nucleosome cores that have been passed through by the transcribing polymerase. Transcription products were analyzed by urea-polyacrylamide gel electrophoresis (Fig. 3). Transcription of *Dra* I-digested free pT207-18 plasmid DNA (lane 1) produces only a single RNA band, as expected for a completely digested free DNA template. As the number of control histone octamers assembled onto these arrays increases (lanes 2–7), a ladder of larger bands appears, corresponding to the passage of RNA polymerase through nucleosome cores

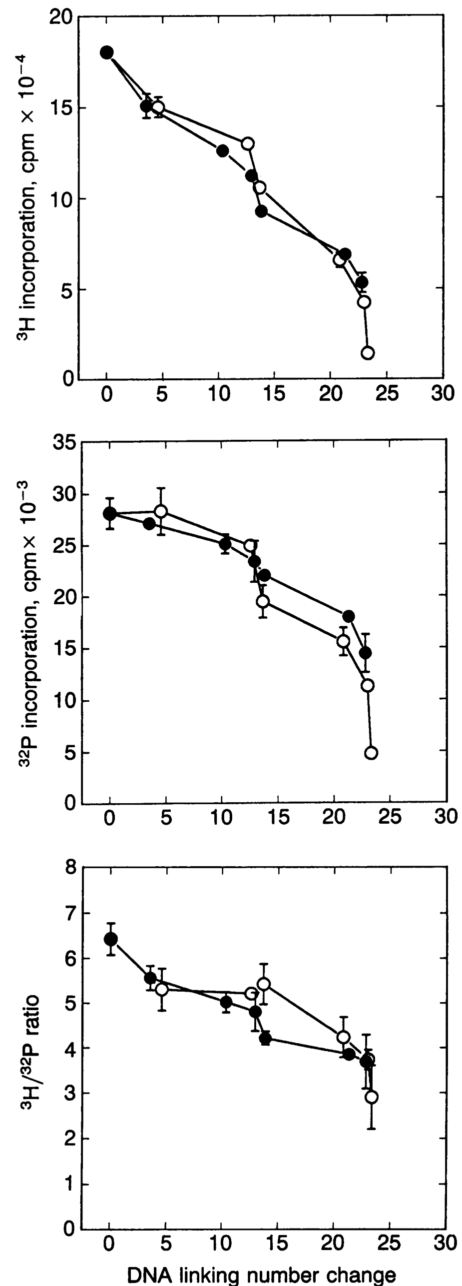

FIG. 2. Effect of crosslinked (●) or control (○) histone octamers transcription of arrays of nucleotide cores with T7 RNA polymerase *in vitro*. In these assays, the incorporation of [^{3}H]UTP into transcripts reflects the level of total nucleotide incorporation (*Top*), the incorporation of [^{32}P-GTP] into transcripts is proportional to the number of transcripts produced (*Middle*), and the ratio of [^{3}H]UTP to [^{32}P]GTP is proportional to the average length of the transcripts (*Bottom*). DNA linking number change is an index of the number of nucleosome cores reconstituted onto the pT207-18 DNA template.

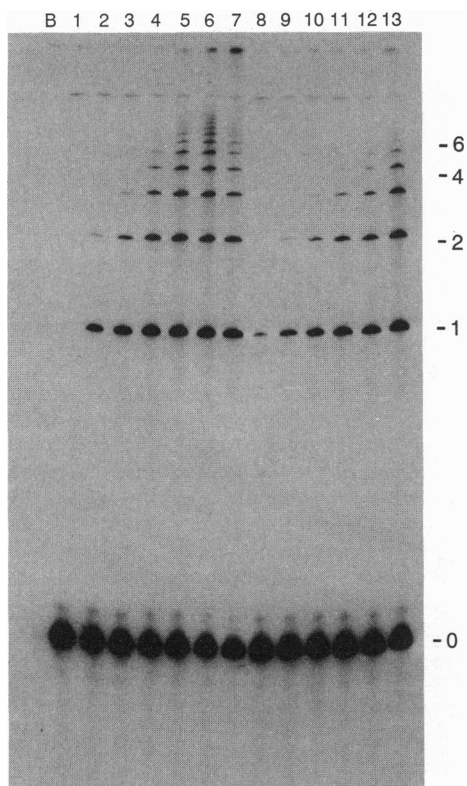

FIG. 3. T7 RNA polymerase transcription through arrays of nucleosome cores reconstituted from control (lanes 1–7) and crosslinked (lanes 8–13) histone octamers. Reconstituted pT207-18 minichromosomes were digested with *Dra* I and were used as templates in transcription reactions with T7 RNA polymerase. Transcripts were analyzed by electrophoresis in 6% polyacrylamide gels containing 8.3 M urea, followed by autoradiography. Lanes: B, blank reaction, no DNA; 1–7, DNA templates onto which approximately 0, 4.6, 12.6, 13.6, 20.8, 23.0, and 23.3 control nucleosome cores have been assembled, respectively; 8–13, DNA templates onto which approximately 3.5, 10.3, 12.9, 13.8, 21.3, and 22.8 nucleosome cores have been assembled from extensively crosslinked histone octamers, respectively. The number of nucleosome cores transcribed through by RNA polymerase is indicated on the right (0–6).

as previously observed (4). Similarly, a ladder of RNA bands appears as increasing numbers of crosslinked histone octamers are assembled onto these arrays (lanes 8–13). Slightly reduced efficiency of synthesis of longer RNA transcripts from crosslinked octamer templates may be a reflection of the greater stability of core particles assembled from dimethyl suberimidate crosslinked histone octamers (19). However, the appearance of a ladder of RNA bands shows clearly that T7 RNA polymerase can transcribe through nucleosome cores reconstituted from both crosslinked and control histone octamers. Hence, dissociation or splitting of the histone octamer is not necessary for passage of RNA polymerase through a nucleosome *in vitro*.

DISCUSSION

In a previous study, we showed that linear arrays of reconstituted nucleosome cores partially inhibit both initiation and elongation by T7 RNA polymerase *in vitro* (4). It was shown that the RNA polymerase can transcribe through arrays of at least 10 nucleosome cores. In the current work, we have shown that the effects of nucleosome cores reconstituted from extensively crosslinked histone octamers on T7 RNA polymerase transcription are very similar to those reconstituted from control histone octamers. Thus, T7 RNA polymerase can transcribe through arrays of nucleosome cores formed from extensively crosslinked histone octamers, demonstrating that the histone octamer need not dissociate, even partially, for the RNA polymerase to elongate through a nucleosome core. These observations provide important insights into one of the central questions in chromatin research today, concerning the mechanism of transcription elongation through nucleosomes.

The fact that transcription occurs through nucleosomes at all is quite remarkable, given the apparent obstacles that a transcribing RNA polymerase must overcome to gain access to the DNA sequences occupied by nucleosome cores. RNA polymerases, even the smallest of which are comparable in size to a complete histone octamer, must be able to access and separate the strands of the DNA duplex despite the steric and topological hindrance of the superhelical coiling of the double helix around the surface of a histone octamer. Clearly, histone–DNA interactions within the nucleosome need to be significantly altered at least transiently to allow passage of the RNA polymerase. Hence, models for transcription through nucleosomes may be divided into two classes—those in which only histone–DNA contacts are affected [class I mechanisms, such as the histone octamer transfer model of Clark and Felsenfeld (13)] and those in which both histone–DNA and histone–histone interactions within the histone octamer are affected [class II mechanisms, such as the half-nucleosome splitting model of Weintraub *et al.* (8) or the progressive displacement model of van Holde *et al.* (12)]. Extensive crosslinking of histones within the histone octamer effectively blocks the possible effects of this latter class of mechanisms in our experiments. Further, the similar behavior of the control and crosslinked octamer-reconstituted nucleosomal templates in these transcription studies indicates that class II mechanisms do not contribute significantly to the transcription of control octamer control nucleosomal templates, if such mechanisms occur at all in this system. We conclude that the dominant mechanisms for transcription through nucleosome cores must involve breakage of bonds between DNA and an intact histone octamer through the action of the elongating RNA polymerase. Hence, the histone octamer as a unit must be at least transiently displaced during transcription through a nucleosome.

Early studies of the transcription of crosslinked histone octamer–DNA complexes gave contradictory results. Wasylyk and Chambon (27) concluded that reconstitution of dimethyl suberimidate crosslinked histone octamers on simian virus 40 DNA had a greater inhibitory effect on nonspecific transcription by *Escherichia coli* RNA polymerase, than did control octamers. However, these experiments were performed under elevated NaCl conditions (0.4–1 M), and total RNA synthesis was measured without distinguishing between the number and length of transcripts produced. Gould *et al.* (28) showed that extensive crosslinking of histones with Lomant's reagent did not affect the length of transcripts produced from linker histone-depleted polynucleosome templates by *E. coli* RNA polymerase. It was concluded that dissociation of the histone octamer does not occur during transcription, in agreement with the current study.

The recent work of Clark and Felsenfeld (14) indicates that *in vitro* transcription through nucleosomes occurs via a nucleosome displacement mechanism. In their studies, a single nucleosome core was assembled onto a specific site within a closed circular plasmid. After transcription through this nucleosome core by SP6 RNA polymerase, it was determined that the histone octamer had been displaced to other DNA binding sites within the plasmid molecule. Because recovery of nucleosomal particles after transcription was high and the movement of the histone octamer was not affected by linearization of the plasmid prior to transcription, the authors concluded that there was no significant loss of histone octamers to solution and that this movement could

not be due to sliding of the histone octamer downstream of the RNA polymerase. In the two remaining possible mechanisms for this movement, which are virtually indistinguishable experimentally, RNA polymerase causes either the direct transfer of the histone octamer from one site to another on the same plasmid molecule or the complete displacement of the histone octamer from its site on the DNA, followed immediately by its free diffusion and binding to another site. The question of whether the histone octamer was transferred as an intact unit was not specifically addressed in these experiments.

Other work supports the case for displacement of nucleosomes by transcription. Lorch *et al.* (1) observed that a histone octamer is displaced from a short linear plasmid DNA fragment upon transcription with either SP6 RNA polymerase or eukaryotic RNA polymerase II. However, Losa and Brown (2) found that the histone composition and position of a histone octamer reconstituted onto a short linear 5S RNA-encoding (rDNA) DNA fragment was not altered upon transcription by SP6 RNA polymerase. The issue was resolved when Lorch *et al.* (29) showed that displacement of the histone octamer during transcription is dependent on the DNA sequence of the transcribed nucleosome core. Because the 5S rDNA sequence has a very high affinity for histone octamers (30), it is possible that histone octamers are displaced upon transcription through nucleosomes on both DNA sequences, but only recaptured on the 5S rDNA sequence. On reconstituted closed circular nucleosomal templates, net loss of nucleosomes due to transcription has not been observed except in the presence of high levels of topoisomerase activity (31). These authors suggest a mechanism wherein nucleosomes are displaced during transcription by positive supercoiling ahead of the advancing RNA polymerase and are rapidly reformed on the negatively supercoiled DNA left in its wake. When high levels of topoisomerase I are present, these negatively supercoiled regions are rapidly relaxed, and nucleosome reformation after the passage of RNA polymerase is no longer favored.

It is possible that a number of different mechanisms may operate under these different experimental scenarios. However, all of these results can be reconciled to a single transcription mechanism whereby the histone octamer as a unit is transiently displaced from its original nucleosome binding site during the passage of RNA polymerase, possibly maintaining a weakened interaction with the same DNA molecule. Nucleosome structure may re-form subsequently, provided an adequate DNA binding site is available. Whether this mechanism plays a major role *in vivo* is currently a matter of speculation. It is possible that histone octamers are displaced as intact units by the action of RNA polymerases and then subsequently undergo partial or complete dissociation, given the instability of histone octamers under physiological ionic conditions (32), followed by stepwise nucleosome reassembly, perhaps as observed after DNA replication (33). This mechanism could explain features observed in active chromatin regions such as histone (H2A-H2B) dimer depletion (11) and exchange of histone (H2A-H2B) dimers due to transcription (33). The resolution of this question awaits further research on *in vivo* systems.

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