

## Coupled-enzymatic Assays for the Rate and Mechanism of DNA Site Exposure in a Nucleosome

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The packaging of DNA in nucleosomes presents obstacles to the action of gene regulatory proteins and polymerases on their natural chromatin substrates. We recently reported that nucleosomes exist in a conformational equilibrium, transiently exposing stretches of their DNA off the histone surface. Such "site exposure" processes potentially provide the needed access of proteins to DNA in chromatin. However, the experiments that reveal site exposure are carried out on timescales of tens of minutes to hours. The actual rates of site exposure are not known. Here we use T7 RNA polymerase and exonuclease III as probes to obtain a more relevant lower bound on the rate of nucleosomal site exposure. We find that the organization of DNA into nucleosomes detectably slows the elongation rate of the polymerase, but that full-length elongation, which requires access to all of the DNA, occurs on the seconds timescale. Independent experiments with exonuclease III, which probes the outermost DNA segments only, similarly show that site exposure in these regions occurs on a timescale of seconds or faster. We conclude that site exposure is sufficiently rapid that it may play a role in the initial binding of regulatory proteins to nucleosomal target sites. These rapid rates argue against a nucleosome sliding model for the mechanism of site exposure. Surprisingly, the measured rates may be too slow to account for the known rates of polymerase elongation *in vivo*. Mechanisms by which polymerase progression through nucleosomes might be catalyzed are discussed.

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### Introduction

The organization of DNA in nucleosomes in eukaryotic chromatin presents obstacles for transcriptional elongation and gene regulation (Felsenfeld, 1996), because each DNA strand in a nucleosome is protected along most of its length in every helical turn: from below by the histone octamer, from above by the complementary DNA strand, and from one or both sides by other stretches of DNA in the same nucleosome (Richmond *et al.*, 1984; Arents & Moudrianakis, 1993). Higher order folding of chromatin (Widom, 1989) further restricts the accessibility of nucleosomal DNA.

Nevertheless, the substrates for polymerase action and gene regulation *in vivo* are chromatin, not naked DNA. Transcription and replication require that RNA and DNA polymerase have uninterrupted access to the full length of individual DNA strands over distances comprising many nucleosomes or an entire chromosome. Similarly, DNA regulatory sequences will sometimes (perhaps often) be occluded by organization into nucleosomes (Polach & Widom, 1995; Lowary and Widom, 1997), yet these sites must be accessible at appropriate times to the regulatory proteins that control genetic activity. Thus, both polymerase elongation and regulatory protein binding require some measure of nucleosome disassembly or conformational transition to expose buried stretches off the surface of the histone octamer. For transcription and replication, the entire length of nucleosomal DNA must be so exposed, although

Abbreviations used: bp, base-pairs; BZA, benzamidine; nt, nucleotides; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

not necessarily all at once; for regulatory protein binding, a stretch of DNA having sufficient length to provide adequate physical space for the bound regulatory protein must be exposed.

We recently reported the discovery of a dynamic behavior intrinsic to nucleosomes that potentially provides this needed access of regulatory proteins and polymerases to their nucleosomal DNA substrates (Polach & Widom, 1995). Using restriction enzyme cleavage as an assay for accessibility, we find that nucleosomes are in a dynamic conformational equilibrium, transiently exposing DNA segments off the histone surface. While exposed, the DNA segments act as though they are naked DNA. The equilibrium constant for site exposure decreases as sites are moved inward toward the dyad axis of symmetry, consistent with a simple uncoiling model for the mechanism of site exposure. The ability of the model to quantitatively account for measured cooperativity in the binding of multiple proteins to sites within one nucleosome provides further support for this uncoiling picture (Polach & Widom, 1996). Figure 1(a) and (b) illustrate how this dynamic conformational equilibrium potentially provides mechanisms for regulatory protein binding and transcriptional elongation, respectively.

An important missing element in the existing data, however, is any information on the rates of site exposure. While our restriction enzyme digestion experiments obey a rapid-pre-equilibrium limit, neither the molar concentrations of the restriction enzymes, their diffusion coefficients, nor their kinetic parameters are known. The actual digestions take place over timescales of tens of minutes to hours. Similarly, previous studies of nucleosome transcription *in vitro* by us and others used either low temperature and low [NTP], or a substitute divalent metal ion, to slow the rate of synchronous transcriptional elongation through a single nucleosome to the minute to tens of minutes timescale (Studitsky *et al.*, 1995; Protacio & Widom, 1996). Consequently, we do not know whether the intrinsic rates of nucleosomal site exposure are fast or slow compared to the natural *in vivo* timescales of biological regulatory decisions (perhaps seconds or minutes) or of polymerase elongation ( $\approx$  six to ten seconds per nucleosome, see Shermoen & O'Farrell, 1992).

In this new study we use T7 RNA polymerase in conditions optimal for the activity of the enzyme to probe nucleosome dynamics on the seconds timescale. Additional studies using exonuclease III provide an alternative method for probing the dynamics of the end-segments of nucleosomal DNA. We find that site exposure is sufficiently rapid that it may play a role in the initial binding of regulatory proteins to nucleosomal target sites. Moreover, these rapid rates argue against a nucleosome sliding model for the mechanism of site exposure. Surprisingly, the measured rates may be too slow to account for the known rates of polymerase elongation *in vivo*. Mechanisms by which

polymerase progression through nucleosomes might be catalyzed are discussed.

## Kinetic Analysis

### T7 RNA polymerase elongation

In contrast to the case of exonuclease III (see below), T7 RNA polymerase is a highly processive enzyme, and polymerase progression proceeds through the nucleosome without a large diminishment in rate from the site exposure/recapture pre-equilibrium. In our earlier studies we found that the rate of polymerase elongation on nucleosomes is essentially identical to that on naked DNA when elongation is artificially slowed (Protacio and Widom, 1996). Slowed elongation provides enhanced sensitivity to any diminishment in rate owing to competition between elongation and DNA "recapture" (i.e. the process in Figure 1 with rate constant  $k_{21}$ ), yet there is no detectable diminishment. Presumably, the high processivity of the enzyme blocks recapture processes, perhaps just by steric hindrance from the bound advancing polymerase (Sousa *et al.*, 1993; Nudler *et al.*, 1996).

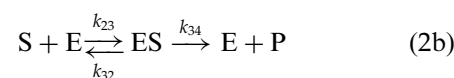
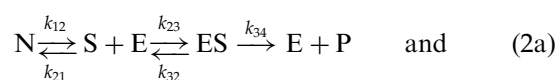
Provided that the polymerase does not contribute to the site exposure process itself (see also Discussion), the rate of polymerase elongation provides a simple lower bound on the rate of site exposure:

$$k_{12} \geq k_{\text{elongation}} \quad (1)$$

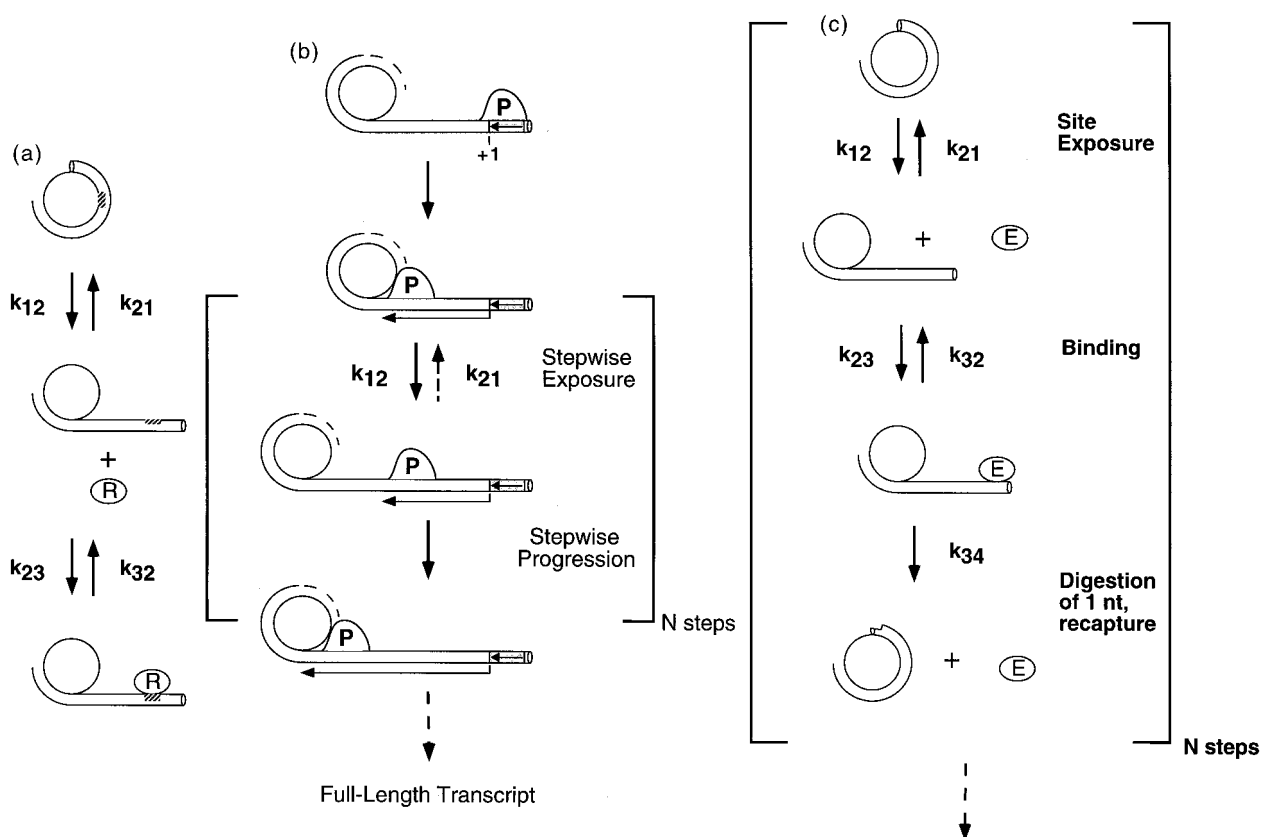
where  $k_{\text{elongation}}$  is the observed rate of elongation on the nucleosomal template.

### Exonuclease III digestion

We take it as a working hypothesis, which will be justified by the experimental results obtained, that the digestion of nucleosomal templates by exonuclease III proceeds in a manner equivalent to that for restriction enzymes which we analyzed previously (Polach and Widom, 1995, 1996). In particular, this requires that release of a stretch of DNA off the histone octamer surface must precede the binding and catalytic action of exonuclease III, and that the digestions proceed in a rapid pre-equilibrium limit. A formal kinetic mechanism corresponding to this case is illustrated in Figure 1(c) and is summarized in equations (2a) and (2b) for nucleosomes and naked DNA, respectively:



where N is the starting nucleosome, S the substrate-active conformation (the nucleosome after site exposure (equation 2a), or naked DNA (equation 2b)), E the enzyme, and P the products.



**Figure 1.** (a) Site-exposure model for the binding of regulatory proteins to nucleosomal target sites. The Figure illustrates how the dynamic property of site exposure, which is intrinsic to nucleosomes, provides a mechanism for the access of regulatory proteins to buried target sites (Polach & Widom, 1995, 1996). In this model, regulatory proteins gain access to their target sites in the exposed state, and bind with an apparent dissociation constant given by their dissociation constant for a naked DNA target site divided by the position-dependent equilibrium constant for site exposure in the nucleosome. This model provides for cooperativity in the binding of multiple regulatory proteins to sites on the same nucleosome, which increases the target-site occupancy achieved for given concentrations of binding proteins. Alternatively, the molecule R could be a protein (or complex) that is capable of "active invasion" of a nucleosome, or it may recruit such a protein to the nucleosome to which it is transiently bound, in either case perhaps leading to displacement of the histone octamer. In these scenarios, the model provides an explanation for how proteins know which nucleosomes to invade. (b) Site-exposure model for the mechanism of polymerase elongation through a nucleosomal template. Successive repetition of two steps (partial uncoiling, followed by elongation up to the next point of steric hindrance) allows full-length elongation. (The octamer could be transferred backward during this process, as has been suggested by Studitsky *et al.*, 1995.) The number of such steps required for elongation through a complete nucleosome in this model depends on the length of DNA released in each step. Our earlier results (Polach & Widom, 1995) establish that site exposure can occur nondissociatively, so the entire DNA chain is not released in just one step; thus the number of site-exposure steps required for full-length nucleosome transcription is two or greater. The structure of the nucleosome suggests that contact points of the DNA and histones (likely sites of histone-DNA "bonds") occur once each DNA helical turn, in which case DNA may be exposed in successive  $\approx 10$  bp-long steps. (c) Site exposure model for the mechanism of Exonuclease III digestion through a nucleosome core particle. Successive repetition of three steps (site exposure, binding of the enzyme, and digestion of nucleotides) results in progressive decrease in the total length of the template. Upon dissociation of the enzyme, the single stranded product might be recaptured by the octamer surface, in which case site exposure must precede successive binding events as well. Since the equilibrium constant for site exposure is position dependent, it is expected that progress of the enzyme will slow as digestion proceeds to more deeply buried nucleosomal sites.

As shown in our earlier analysis, for typical experimental conditions (both  $[S]$  and  $[E_0] \ll K_m$ , where  $[S]$  is the substrate concentration (i.e. after site exposure),  $[E_0]$  the total enzyme concentration, and  $K_m \equiv ((k_{32} + k_{34})/k_{23})$ , the apparent first-order rate constant ( $k_{obs}^{nucleosome}$ ) for digestion of the nucleosomes (N) is given by:

$$k_{obs}^{nucleosome} = \frac{k_{34}[E_0]}{K_m} K_{eq}^{conf} \quad (3)$$

where,  $K_{eq}^{conf} = k_{12}/k_{21}$  is the equilibrium constant for site exposure.

If, in separate experiments, naked DNA is digested under identical solution conditions (but

possibly with different  $[E_0]$ , the reactant naked DNA will disappear with an apparent first-order rate constant given by:

$$k_{\text{obs}}^{\text{naked DNA}} = \frac{k_{34}[E_0]}{K_m} \quad (4)$$

Combining equations (2) and (3) yields:

$$K_{\text{eq}}^{\text{conf}} = \frac{k_{\text{obs}}^{\text{nucleosome}}/[E_0^{\text{nucleosome}}]}{k_{\text{obs}}^{\text{naked DNA}}/[E_0^{\text{naked DNA}}]} \quad (5)$$

Thus, the rate of digestion of the nucleosomes is diminished relative to that of naked DNA by a factor equal to the equilibrium constant for site exposure,  $K_{\text{eq}}^{\text{conf}}$ .

Importantly, exonuclease III is sufficiently active such that the net rate of digestion of nucleosomes,  $k_{\text{obs}}^{\text{nucleosomes}}$ , can occur on the seconds timescale. This allows us to place a relatively rapid lower-limit on the rate constant for site exposure,  $k_{12}$ , as follows.

Since:

$$\frac{k_{34}[E_0]}{K_m} \leq k_{23}[E_0] \quad (6)$$

it follows from equation (2) that:

$$k_{\text{obs}}^{\text{nucleosome}} \leq k_{23}[E_0] \frac{k_{12}}{k_{21}} \quad (7)$$

For the rapid pre-equilibrium mechanism to apply, it is necessary that  $k_{21} \gg k_{23} [E_0]$ ; an appropriate lower bound would be that  $k_{21} \geq 10 k_{23} [E_0]$ . (Note that this is a conservative estimate.  $k_{23}[E_0]$  is unlikely to exceed  $\approx 10 \text{ s}^{-1}$ , whereas simple theoretical models for the site exposure and recapture process, assuming either an activated or a diffusive process for recapture, lead to the expectation that  $k_{21} \geq 10^5 \text{ s}^{-1}$ ; J. Widom, unpublished.) Taking this conservative lower bound leads to  $k_{\text{obs}}^{\text{nucleosome}} \leq 0.1 k_{12}$ , or:

$$k_{12} \geq 10 k_{\text{obs}}^{\text{nucleosome}} \quad (8)$$

and possibly much greater.

## Results

### Experimental strategy: studies with T7 RNA polymerase

In our earlier study of nucleosome transcription by T7 RNA polymerase, we slowed the elongation rate to the minutes timescale, and found that there was essentially no difference in elongation rate on naked DNA or nucleosomal templates (Protacio & Widom, 1996; and see Studitsky *et al.*, 1995). In optimal conditions this polymerase requires only  $\approx$  one to two seconds to transcribe a  $\approx 200$  bp naked DNA template (Golomb & Chamberlin, 1974 and see below). We now ask whether the organization of DNA into nucleosomes detectably slows the elongation of the polymerase on this

much faster and biologically relevant timescale. Complexes of stalled T7 RNA polymerase on nucleosomal or naked DNA templates are prepared in reactions having optimal conditions except for the omission of UTP (Protacio & Widom, 1996). Elongation is synchronously restarted by rapid addition of UTP, and the reactions are quenched after variable delay times of one second to five minutes.

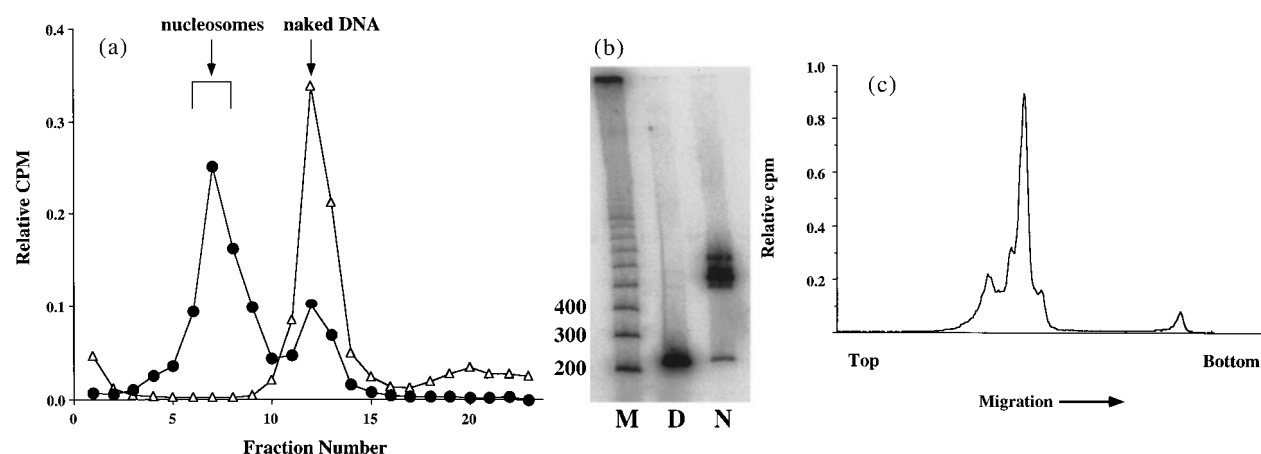
The premise underlying this experiment is that T7 RNA polymerase cannot pass ghost-like through the atoms of the histone octamer and the non-template DNA strand; rather, release of DNA off from the surface of the histone octamer, perhaps as illustrated in Figure 1(b), must precede or accompany forward motion of the polymerase. The question then arises whether polymerase elongation contributes to the mechanism and rate of site exposure; if it does not contribute, then the rate of elongation sets a lower bound on the intrinsic rate of site exposure.

Polymerase progression does provide a thermodynamic driving force for displacement of DNA from the surface of the histone octamer because the polymerase's substrate is naked DNA. However, that is not the relevant question. The important question here is whether the polymerase actively contributes to the displacement process itself, enhancing the rate of displacement, or whether it only traps the displaced state, preventing recapture? The restriction enzymes used as probes of site exposure equilibria in our previous studies also provide a driving force for site exposure; nevertheless, those studies demonstrated that the restriction enzymes serve as neutral probes of site exposure, and do not contribute to the mechanism (or rate constants) of site exposure.

For reasons summarized in Discussion, we conclude that T7 RNA polymerase most likely does not contribute detectably to the rate or mechanism of site exposure. In the worst case, molecular motor behavior of the polymerase augments the natural, spontaneous, thermally driven site-exposure processes, and enhances by at most a few-fold the rates of the site exposure processes that it is helping us to measure. In either case, displacement of DNA segments from the surface of the histone octamer is a consequence of spontaneous thermal fluctuations.

### Purification and characterization of templates

The nucleosomal templates for both assays are prepared from purified histone octamer and a small molar excess of template DNA by stepwise dialysis from 2.0 M NaCl, and are purified by sucrose gradient ultracentrifugation. An example of such a separation is shown in Figure 2(a). This procedure effectively separates reconstituted nucleosomes from contaminating naked DNA. Native gel electrophoresis of the purified nucleosomes (Figure 2(b) and (c)) reveals that naked DNA comprises a maximum of  $4.1(\pm 0.2)\%$  of the



**Figure 2.** (a) Sucrose gradient purification of reconstituted mononucleosomes. Mononucleosomes were separated from aggregates and unincorporated DNA by ultracentrifugation on linear 5% to 30% (w/v) sucrose gradients at 41,000 rpm for 24 hours using a Beckman SW41 rotor. A naked DNA sample was run in parallel on an identical gradient. Following fractionation, each 0.5 ml fraction was analyzed by scintillation counting. The relative counts per minute (cpm) of each fraction for naked DNA ( $\Delta$ ) and the reconstituted nucleosomes ( $\bullet$ ) are plotted against fraction number. (b) Native gel electrophoretic analysis of nucleosomes purified by sucrose gradient ultracentrifugation; a phosphorimage of the gel is shown. M, 100 bp ladder (Pharmacia); D, naked DNA; N, reconstituted nucleosomes. (c) Densitometer trace of lane N from (b). Relative cpm is plotted *versus* migration distance from the top of the gel.

total DNA in each of three samples (and some of this may be an artifactual consequence of the electrophoresis procedure rather than a real property of the sample). The densitometer traces also reveal the presence of positioning isomers, with the major fraction comprising  $87(\pm 1)\%$  of the population. High resolution mapping using micrococcal nuclease digestion and primer extension methods (Protacio and Widom, 1996) reveals that the dominant population is positioned at the desired region of the template, which also corresponds to the site defined in our previous studies using this sequence (Polach & Widom, 1995; Protacio & Widom, 1996); the minor alternative positions are displaced toward the T7 promoter-proximal end of the DNA.

### Full-length nucleosome transcription on the seconds timescale

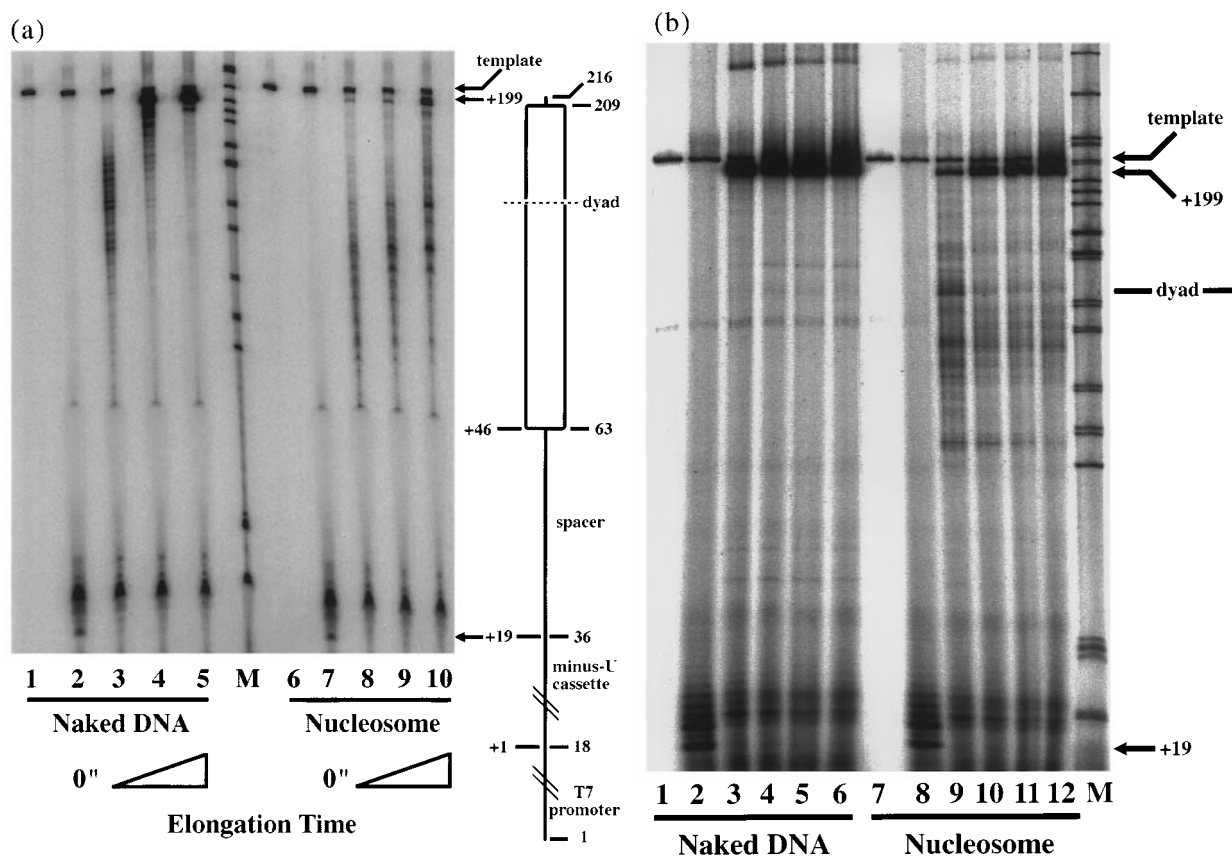
Stalled transcription complexes are prepared using naked DNA and nucleosomal templates; elongation is restarted by rapid injection of UTP, and the reactions are quenched, after differing times of elongation, by injection of EDTA. Control experiments in which EDTA is injected prior to UTP establish the efficacy of the quenching procedure (data not shown). The transcription reactions were run on denaturing 6% polyacrylamide sequencing-size gels to analyze the products at nucleotide resolution. The stalled RNAs and chased-out transcripts from both naked DNA and nucleosomal templates from a typical experiment are shown in Figure 3(a). For each set of reactions, the bands are clearly transcription dependent: the control lanes containing template alone reveal a

single band corresponding to the radiolabeled DNA construct.

As we noted previously (Protacio & Widom, 1996), the stalled transcripts which are expected to be uniquely 19 nt in length are actually heterogeneous and composed of products 19 nts and longer. The extra bands evidently originate from the incorporation of extra nucleotides despite the construct design, either due to UTP contamination in the stalling-reaction mixture or to misincorporation of incorrect NTPs (Studitsky *et al.*, 1995; Protacio & Widom, 1996).

Reinitiation is not expected to occur significantly for the restricted period analyzed in this experiment, because initiation is slow even in optimal conditions (Chamberlin & Ring, 1973). In addition, it is clear from the appearance of the gel tracks that intermediate-sized transcripts (representing sites of preferential pausing; see below) do not re-emerge once they have chased-out to full length. We conclude that only the first passage is examined in this experiment.

The efficiencies of initiation, chase-out, and completion for naked DNA and the nucleosomal template have been quantified and are given in Table 1. Approximately 100% of the naked DNA templates and the majority of the nucleosomal templates (83%) participate in the reactions. By the four-second final timepoint of the experiment in Figure 3(a), 14% of the nucleosomal templates have completed full-length transcription. This fraction is much greater than the maximal contamination from free DNA, so it necessarily arises from transcription of nucleosomal templates. We conclude that full-length nucleosome transcription can occur on the seconds timescale.



**Figure 3.** Kinetics of transcriptional elongation. (a) Aliquots for short elongation times from reactions on naked DNA (lanes 2 to 5) or nucleosomal templates (lanes 7 to 10) were run on sequencing-size 8 M urea/6% polyacrylamide gels and analyzed with a phosphorimager. Control lanes containing template alone are shown for naked DNA (lane 1) and the purified nucleosomes (lane 6). Samples were taken after stalled complex formation (lanes 2 and 7), and after one (lanes 3 and 8), two (lanes 4 and 9) or four seconds (lanes 5 and 10) of elongation following UTP addition. Markers (lane M) are *MspI*-digested pBR322 fragments (New England Biolabs). The diagram on the right is a representation of the template with the region organized in the nucleosome core particle portrayed as an open box. Numerical labels to the right of the diagram refer to nucleotide positions on the template. Numbers to the left of the diagram refer to the corresponding transcript lengths. The arrows identify the stalled complex (+19), the full length transcript (+199) and the radiolabeled template on the gel. (b) Aliquots for long elongation times from reactions on naked DNA (lanes 1 to 6) or nucleosomal templates (lanes 7 to 12) were also run on sequencing-size 8 M urea/6% polyacrylamide gels and analyzed with a phosphorimager. Control lanes containing template alone are shown for naked DNA (lane 1) and the purified nucleosomes (lane 7). Samples were taken after stalled complex formation (lanes 2 and 8), and after four seconds (lanes 3 and 9), one (lanes 4 and 10), two (lanes 5 and 11) or five minutes (lanes 6 and 12) of elongation following UTP addition.

### Efficient completion of full-length transcription

We wished to extend the elongation reactions to longer times to verify that most nucleosomal templates are capable of completion. In our earlier studies, we found that when transcription is artificially slowed to the minutes or tens of minutes timescale, the efficiency of completion is very high (45 to 75% on a minute-timescale, and 81 to 98% on a ten minute timescale; Protacio and Widom, 1996). However, since the present studies are carried out in different solution conditions, the fraction of templates capable of completion is not necessarily equally high.

Extending the reactions to longer times in this case is complicated by the possibility of re-

initiation, which may be facile in optimal conditions. To simplify the analysis, elongation was recommenced with the addition of a great excess of cold ATP such that the probability of incorporation of [ $\alpha$ - $^{32}$ P]ATP molecules after restarting is reduced 800-fold. Furthermore, the low concentration of UTP in the elongation reaction (60  $\mu$ M) limited the polymerases to at most  $\approx$ 23 rounds of transcription. Thus, the specific activity of transcripts from the first round of transcription will vary little with length, and products from subsequent rounds will incorporate little additional label.

A representative set of reactions are shown in Figure 3(b). For the naked DNA templates, radiolabeled transcription is effectively over by four

**Table 1.** Efficiencies of initiation, chase-out and completion for short elongation times

Efficiency	Naked DNA	Nucleosome
Initiation	1.00 <sup>a</sup>	0.83
Chase-out	0.25	0.41
Completion		
one second	0.00	0.02
two seconds	0.84	0.02
four seconds	1.20	0.14

<sup>a</sup> The efficiencies of initiation, chase-out and completion were calculated as follows. Our previous studies demonstrated that the absolute efficiency of utilization of the naked DNA template in this system is approximately 100% (Protacio & Widom, 1996); thus, the efficiency of initiation for naked DNA is defined here as 1.00. Initiation efficiency for the nucleosomal template was then determined by measuring the number of counts in the stalled transcripts (19 nts and longer) and dividing it by the stalled counts from naked DNA. Chase-out efficiency was calculated by comparing the number of counts in the original stalled product to the number of stalled product counts remaining in the restarted reactions. The efficiencies of completion were calculated by comparing the number of counts in the full-length transcript present at different elongation times to the full-length counts expected from the stalled complex counts that successfully chased out. The calculations take into consideration changes in product specific activity based on the increasing number of As in the transcript with length and the decreasing radiolabel concentration with variations in volume.

seconds. The counts in the full-length band increase slightly over time, but the average increase by five minutes of elongation time for three independent experiments was 35%, in reasonable accord with a predicted 26% increase for  $\approx 23$  additional rounds of transcription; moreover, some of this increase is attributable to a fraction of molecules having particularly long pause times during their first passage. Evidently, only the first passage is significantly labeled and examined in this experiment.

For the nucleosomal templates, the qualitative appearance of the gel demonstrates the disappearance of paused intermediate-length complexes and the accumulation of full-length counts beyond the final four seconds of elongation time of the experiment in Figure 3(a). Intermediate-sized transcripts do not become re-populated once they have chased-out. The relative completion efficiencies are measured by comparing the full-length counts produced from the nucleosomal templates at each elongation time to the full-length counts produced from naked DNA at four seconds of elongation, at which time the first round of transcription for the naked DNA is essentially complete and there is little additional incorporation from reinitiation. The quantitative results are summarized in Table 2, and confirm the qualitative interpretation. There is good agreement in completion levels at four seconds between the two sets of experiments, despite the technical differences in set-up and execution. We conclude that the majority of the transcribing complexes are not permanently stalled and do eventually reach full length.

**Table 2.** Efficiencies of completion on nucleosomal templates for longer elongation times

Elongation time (seconds)	Relative completion (%)	Corrected completion (%)
4	9.5 $\pm$ 1.8	12.3 $\pm$ 2.4
60	24.0 $\pm$ 0.7	31.1 $\pm$ 0.6
120	33.0 $\pm$ 0.1	42.7 $\pm$ 0.5
300	40.7 $\pm$ 0.2	52.8 $\pm$ 0.5

Relative completion and the standard deviation for each time point were calculated by measuring the full-length transcript counts produced from the nucleosomal template and expressing that as a fraction of the full-length counts transcribed from the naked DNA template after the first round of transcription. Corrected completion corrects for differences in the amount of stalled complexes formed from the two templates that chase-out upon addition of UTP.

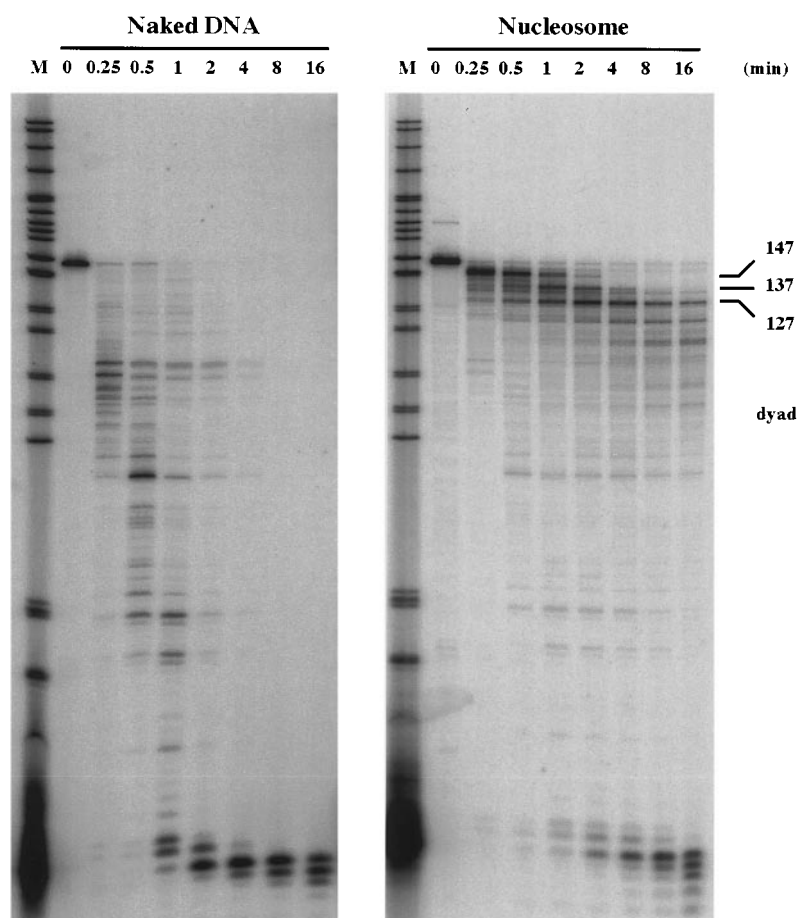
### Experimental strategy: studies with exonuclease III

We developed an alternative assay for the rate of site exposure, using exonuclease III as a probe. This experiment (Figure 1(c)) is closely analogous to our prior studies using restriction enzymes as probes. As will be seen below, the rate of exonuclease III digestion on nucleosomal templates compared to the rate on naked DNA provides a measure of the equilibrium constant for site exposure, in quantitative agreement with those earlier studies. However, unlike for the restriction enzymes, available exonuclease III activities are sufficiently great that this enzyme can also be used to place reasonably short time limits on the rate of site exposure.

Compared to the approach using T7 RNA polymerase, these studies using exonuclease III have both advantages and disadvantages. The chief advantage is that this enzyme is less subject to a concern that it actively contributes to site exposure: it is less credible to suppose that exonuclease III is a molecular motor capable of converting the chemical energy of polynucleotide hydrolysis into mechanical work to actively displace DNA from the histone octamer, speeding up the rate of site exposure. (Again, one must not confuse the provision of a thermodynamic driving force to active participation in the rate-limiting kinetic step.) A chief disadvantage is that after sufficient digestion (in principle, as little as the first nucleotide inside the core particle) the nucleosomes may become unstable and the rate of digestion no longer reflects the rate of site exposure. Thus, only the outermost segments can be reliably probed.

### Purification and characterization of templates

The templates used for these studies resemble those used for our earlier studies of restriction enzyme digestion. Like the templates used in the transcription studies above, they derive from the 5 S gene nucleosome positioning sequence; but the length is shorter, 155 bp in the present case. The shorter length suppresses multiple positions



**Figure 4.** Exonuclease III digestion of naked DNA and reconstituted nucleosome core particles. Aliquots from digestion reactions on naked DNA (left panel) and nucleosomal templates (right panel) were run on 8% polyacrylamide DNA sequencing gels. Samples were taken prior to initiation (0 minutes) and at 0.25, 0.5, one, two, four, eight and 16 minutes after addition of the enzyme. Markers are *Msp*I-digested pBR322 fragments (New England Biolabs). Strong pause sites in the nucleosome digestions are marked in base pairs on the right along with the approximate position of the dyad axis of symmetry.

(Lowary and Widom, 1997), so that the desired positioning is even more strongly populated (Polach and Widom, 1995). The DNA is labeled on one strand, at the 5' phosphate. Nucleosomes are reconstituted and purified using methods identical to those used for the transcription experiments. Native gel analysis reveals similar levels of purity of the reconstituted nucleosomes to those shown in Figure 2, and further suggests that positioning isomers are largely absent (data not shown).

### Rapid digestion by exonuclease III

Purified reconstituted core particles and naked DNA are incubated with exonuclease III at 37°C; at various times aliquots are withdrawn, and the reactions quenched. Digestion by the exonuclease leads to progressive shortening of the radiolabeled DNA strand. The extent of digestion by the enzyme at each timepoint is assessed by electrophoresis on a sequencing gel and quantified using a phosphorimager. The results of one such experiment using identical conditions and enzyme concentrations for nucleosomal and naked DNA templates are shown in Figure 4.

For the nucleosomal DNA template, the starting 155 bp DNA is very rapidly digested to the 147 bp core particle, but the next stage of digestion, down to the next transient pause site at 137 bp, is mark-

edly slowed in comparison to the rate on naked DNA. This finding is consistent with the hypothesis that digestion of the core particle DNA is diminished in rate by the site-exposure pre-equilibrium constant, as illustrated in Figure 1(c). There is very little population of intermediates between 147 bp and 137 bp length. The corresponding first order rate constant for loss of the 147 bp core particle "reactant"  $\equiv k_{\text{obs}}^{\text{nucleosome}} = 0.013 \text{ s}^{-1}$ . The 137 bp particle is further digested to a variety of shorter intermediates over a longer timescale. This observation is also consistent with a required site-exposure pre-equilibrium, since we find that the equilibrium constants for site exposure decrease progressively for sites further in toward the nucleosomal dyad (Polach & Widom, 1995).

Using the value  $k_{\text{obs}}^{\text{nucleosome}} = 0.013 \text{ s}^{-1}$ , equation (8) yields a lower bound on the rate constant of site exposure of the outermost DNA segment,  $k_{12} \geq 0.13 \text{ s}^{-1}$ , corresponding to a time constant  $\tau = k_{12}^{-1} \leq \text{eight seconds}$ . We emphasize that  $\tau$  may actually be *much* shorter (i.e.  $k_{12}$  much faster); this experiment only provides a bound.

### Discussion

At present we have no assay available that would allow direct detection of site exposure events. Nevertheless, it is important to learn about

the rates. In particular, we need to determine whether the rates are fast or slow compared to the timescales of key biological regulatory processes, which may be seconds to minutes. For this reason, we use an alternative approach, coupling the site exposure process which we cannot detect to either of two different enzymatic processes, which do yield detectable products. The underlying idea is that simply because of steric hindrance, elongation by T7 RNA polymerase (Sousa *et al.*, 1993) or exonuclease III (Mol *et al.*, 1995) cannot take place when DNA is wrapped on the histone surface of the nucleosome. Rather, we suppose that exposure of the DNA off of the histone surface must accompany or precede the action of these enzymes. If (and only if) these enzymes do not contribute to the rate of site exposure, then the measured rates of the enzymatic reactions which depend on site exposure allow us to place lower bounds on the rate constants for site exposure, defined as  $k_{12}$  in Figure 1(a) to (c).

### Can T7 RNA polymerase influence the rate of site exposure?

For the experiments using T7 RNA polymerase, a particular concern is that a molecular motor-like property of the polymerase could contribute to the rate of site exposure by actively prying DNA off the nucleosome, thereby leading us to overestimate the rates intrinsic to nucleosomes. However, several lines of reasoning argue against this possibility.

First, while molecular motor proteins do act effectively as power-driven levers, it is by no means established that they act in that manner at the level of their detailed molecular mechanism. Rather, many investigators consider that molecular motors act via "thermal ratchet" mechanisms, using the energy of NTP hydrolysis to trap successive states that are reached by spontaneous (not power-driven) thermal fluctuations (Meister *et al.*, 1989; Astumian, 1997).

Secondly, suppose that molecular motor proteins actually do act as power-driven levers. The following argument reveals that the related RNA polymerase from *Escherichia coli* (currently the strongest molecular motor known) lacks the force by a factor of ten to break even individual, unnaturally weak salt-bridges (bonds thought to hold DNA on the nucleosome; Arents and Moudrianakis, 1993), and can contribute at most negligibly or only a few-fold (in the weak-bonding and strong-bonding limits, respectively) to the rate at which DNA would be released in thermally driven (i.e. spontaneous) site-exposure processes.

Let us define an unnaturally "soft" ionic interaction such that it takes  $1 \text{ kcal mol}^{-1}$  of energy to stretch the bond by  $1 \text{ \AA}$ , and there is a quadratic dependence of energy on displacement. (Real ionic interactions may have considerably higher energies and will reach those energies with much smaller displacements; hence real ionic interactions are

much stiffer, requiring much more force to stretch, than this hypothetical one.) The force required to stretch this soft hypothetical bond, raising its energy by this small amount, is given by the derivative of the energy with displacement, which equates to  $2 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ , or 140 pN, ten times the maximal force measured for RNA polymerase (Yin *et al.*, 1995). Even if the entire force of the RNA polymerase could be directed to prying DNA off the histone octamer, it lacks the force by a very large margin to break even a single unnaturally weak bond. We conclude that RNA polymerase does not cause the site exposure processes that it is allowing us to detect. The site exposure processes necessary to allow polymerase progression arise from spontaneous thermal fluctuations, as must be the case for restriction enzyme and exonuclease III digestion experiments.

Thirdly, we consider to what extent the polymerase's motor force could significantly alter the rate of spontaneous thermally driven site exposure if this entire motor-force were directed to stretching a bond holding DNA onto the nucleosome. Two limits need to be considered. (i) If the free energy barrier to site exposure is low to fairly large ( $\leq \approx 15 \text{ kcal mol}^{-1}$ ), then any effect due to the polymerase would be negligible because the frequency of thermal fluctuations having or exceeding that energy (estimated from transition state theory) greatly exceeds the actual elongation rate of the polymerase. In other words, for such barriers, uncatalyzed site exposure is already rapid in comparison to the forward motion of the polymerase. (ii) If instead the free energy barrier is extremely large ( $\geq 15 \text{ kcal mol}^{-1}$ ), then any ability of the polymerase to stretch the bond holding DNA onto the nucleosome will contribute to the rate of site exposure. We can estimate this effect as follows. Suppose that the polymerase can exert its maximal force of 14 pN over its entire working stroke, which we take to be  $3.4 \text{ \AA}$  (Yin *et al.*, 1995). An upper bound on the work done by the polymerase is then given by the product of the force and displacement,  $= 0.68 \text{ kcal mol}^{-1}$ . Suppose that all of this work could be channeled instead into stretching the bond holding DNA onto the nucleosome. This would reduce the energy of thermal fluctuations needed to break the bond by  $0.68 \text{ kcal mol}^{-1}$ , which would increase the frequency of thermal fluctuations having the necessary energy by the factor  $\exp(0.68/RT)$ , i.e.  $\approx$  threefold. Thus, in this high-barrier limit, the polymerase could conceivably actively contribute to enhancing the rate of site exposure, but only by  $\approx$  threefold at the most, and almost certainly less, since it is unlikely that the polymerase's entire mechanical work could be directed toward this action.

In summary, a molecular motor action of the polymerase lacks the energy to force site exposure on its own, and can contribute only negligibly or, at the extreme, at most  $\approx$  threefold, to the rate of spontaneous thermally driven site exposure.

Finally, other potential mechanisms whereby T7 RNA polymerase might contribute to the site exposure process itself also seem unlikely. The polymerase does, in a sense, have a helicase-like activity which might be imagined able to unwind nucleosomal DNA ahead of the polymerase; however, the structure and properties of this and related polymerases suggests that their leading (forward) domain contacts and stabilizes a double-stranded template, not a single-stranded one (Sousa *et al.*, 1993; Nudler *et al.*, 1996).

### Rate of site exposure as monitored by the coupled transcription assay

Our rapid transcription experiments (Figure 3(a)) demonstrate that site exposure sufficient to allow full-length transcription on a nucleosomal template, which requires access to the entire length of the nucleosomal DNA, can occur on the seconds timescale. Direct catalysis of site exposure by the polymerase is unlikely and could enhance the measured rates by threefold at the most. Thus, site exposure processes can occur on the seconds timescale or faster. Taking the value 14% completion at a time of two seconds (beyond that required on naked DNA) corresponds to a time constant  $\tau \approx 13$  seconds. Since site exposure is non-dissociative (Polach and Widom, 1995), access to the full DNA length requires at least two such events, which, if they have equal rates, yields  $\tau \approx$  seven seconds. (Again, it is very likely that the rate-limiting step is the polymerase chemical reaction cycle, not site exposure, so these values for  $\tau$  represent upper bounds on the corresponding time constants for site exposure).

It has been suggested that after a polymerase reaches the nucleosomal dyad, the histone octamer may be transferred backwards onto the already-transcribed DNA behind the polymerase, so that further transcription beyond the original nucleosomal dyad may actually take place on naked DNA (Studitsky *et al.*, 1995; and see below). Thus, a more accurate interpretation of our new results is that site exposure adequate to expose all of the DNA up to the dyad axis occurs on the seconds timescale. Note that exposure of DNA from one end in to the dyad suffices to allow the binding of regulatory proteins anywhere within the nucleosome, since site exposure occurs spontaneously on both sides of (dyad-symmetric) nucleosomes.

Figure 3(b) and Table 2 reveal that a significant fraction of the nucleosomal templates take rather longer to reach full length. If there were a single process having  $\tau \approx 13$  seconds, then it should be essentially complete by 60 seconds; however, at that point only  $\approx 31\%$  of the active templates have reached full length. An additional  $\approx 22\%$  take somewhat longer, and the remainder take longer still or may never reach completion. Which of these behaviors are typical? Should we instead be concluding that site exposure is a slow process?

The methods used to produce and purify the starting nucleosomes yield native-like nucleosomes. The DNA is homogeneous, and the core histones which are isolated from chicken erythrocytes are also essentially homogeneous (diverse post-translationally-modified states are largely absent). The only source of significant heterogeneity that we detect arises from a subpopulation ( $\leq 13(\pm 1)\%$ ) having alternative positionings of the histone octamer on the DNA, which we have characterized (Protacio and Widom, 1996; and see Results). Differing positionings can affect the ability to initiate transcription if some positions occlude the promoter, but are not known to affect transcription rates once transcription is initiated (Lorch *et al.*, 1987; Wolffe & Drew, 1989). We find that when transcription is carried out on a slower timescale, essentially all of the nucleosomes that allow transcriptional initiation also allow elongation to full length, with no detectable slowing relative to the rate on naked DNA in the same conditions (Protacio and Widom, 1996).

We believe that the fraction of nucleosomal templates that take much longer or fail to ever reach full length in the rapid reactions are a consequence of non-nucleosomal structures induced in a fraction of the templates by the process of transcription itself. Native gel analysis of the template-DNA containing species present at the completion of the first round of nucleosome transcription reactions (even in the artificially slowed reactions) reveals that transcription induces the presence of significant amounts of naked DNA and new DNA-containing nonnucleosomal species (data not shown). Histones have a very high affinity for DNA in approximately physiological conditions, so histones that were originally bound to the now-naked DNA have almost certainly bound to DNA on other intact or partially-disassembled nucleosomes (Voordouw & Eisenberg, 1978; Gallego, *et al.*, 1995). Such non-nucleosomal structures could easily inhibit polymerase progression. The fraction of templates in the rapid reactions that never chase to full length even after five minutes (Table 2) is substantially greater than the fraction that fail to chase-out when transcription is artificially slowed to the ten-minute timescale (Protacio & Widom, 1996). If our explanation of non-nucleosomal structure formation is correct, then we would conclude that the extent to which such nonnucleosomal structures are formed depends on the rate of transcription, and is increased when transcription is especially rapid. Additional indications that the structural outcome of nucleosome transcription does depend on transcription rate are discussed below.

Finally, we note that while full-length transcription of nucleosomal DNA does occur on the seconds timescale, transcription of the nucleosomal template is nevertheless significantly slowed relative to transcription on a naked DNA template in identical conditions. This result stands in marked contrast to our earlier results obtained with the

same system except with elongation slowed to one to ten-minute timescales, for which we found that elongation rates on nucleosomal and naked DNA templates were essentially identical. Taken together these data imply that the organization of DNA into nucleosomes measurably decreases the rate of T7 RNA polymerase progression on the seconds timescale. We attribute the increased time required for nucleosome transcription to nucleosome-specific dynamics such as required steps of site exposure, or other effects, discussed below, such as "friction" of the polymerase with the histones.

### Increased pausing and an altered pause-site distribution during rapid nucleosome transcription

A striking feature of these reactions is the effects of polymerase pausing, which is responsible for increasing the spread of transcript sizes above that expected for stochastic polymerization alone and, moreover, causes the transient accumulation of intermediate-length transcripts at discreet sizes. The one-second elongation reactions for naked DNA and the nucleosomal template were run side-by-side to facilitate a more detailed comparison of the pause patterns (data not shown). The pause sites detected on both naked DNA and nucleosomal templates are identical, but the bands representing these sites are increased in intensity on the nucleosomal template. This confirms the surprising previous finding that the organization of DNA into nucleosomes increases the residence times at pause-sites that are intrinsic to the DNA sequence (Studitsky *et al.*, 1995; Protacio & Widom, 1996; and references therein).

Interestingly, comparison with our earlier results shows that pause sites are relatively much more prominent in these rapid reactions than in our previous studies of much (30 to 150 $\times$ ) slower reactions. More remarkably, these new data for the rapid reactions show that pausing on the nucleosomal templates occurs in packets. There is enhanced pausing at *sets* of nucleotides, with the centroid of each packet corresponding to the pause site on naked DNA. In each of these respects, the system acts as though there is "friction" between the elongating polymerase and the residual nucleosome. Such behavior might be expected if site-exposure events occur in short stretches, so that the polymerase is never far from the histones (or from other stretches of DNA in the same nucleosome). Alternatively, perhaps DNA segments in the exposed state may not be fully equivalent to naked DNA; e.g. perhaps interactions with histone tails are maintained and these limit polymerase progression.

Another noteworthy aspect of the pause site distribution in these rapid reactions is that pausing on the second half of the nucleosomes is greatly reduced compared to our previous results on slower timescales. Although we can still locate the bands corresponding to three significant pause

sites in the downstream half of the nucleosome (designated p10, p11 and p12 in our previous study), they are quite faint and diminished in relative intensity. This is consistent with the findings of another study, in which the authors suggest that the histone octamer may be transferred behind the progressing polymerase, so that elongation through the second half of the nucleosome may be occurring on naked DNA (Studitsky *et al.*, 1995). In combination with our earlier results (Protacio & Widom, 1996), these new results imply that the structural outcome of nucleosome transcription *in vitro* depends on the relative rate of polymerase elongation.

### Site exposure and gene regulation

One conclusion of this study is that the exposure of nucleosomal DNA segments to an extent adequate to allow access everywhere to T7 RNA polymerase, which we presume also to suffice for simple binding by regulatory proteins (or restriction enzymes), is intrinsic to nucleosomes in physiological conditions, and can occur on a timescale of seconds or faster. This conclusion is confirmed at least for the outermost nucleosomal DNA segments by independent experiments using exonuclease III.

We previously proposed that site exposure provides a mechanism whereby gene regulatory proteins may be guaranteed access to their DNA target sequences in chromatin *in vivo* (Polach and Widom, 1995). While regulatory protein binding might be catalyzed *in vivo* (see below), an unanswered question is how such a hypothetical catalyst might know where in the genome to function. One could invoke another prior-bound protein, but this simply replicates the fundamental problem one level up in a regulatory hierarchy. The simplest mechanism is one in which an uncatalyzed site exposure process provides the needed initial access of regulatory proteins to their target sites; then, once initial site recognition is achieved (possibly involving the cooperative binding of multiple proteins; see Polach & Widom, 1996), more stable regulatory complexes may be formed with the subsequent (and possibly catalytic) displacement or modification of the histone octamer. The present results establish that uncatalyzed nucleosomal site exposure occurs rapidly enough that this mechanism may be feasible.

It remains to be tested how the folding of a nucleosome filament into higher order chromatin structures may affect the rate of site exposure. Importantly, the available evidence suggests that the 30 nm chromatin fiber (the next higher level of structure) is only marginally stable and is itself in dynamic equilibrium with extended states (Widom, 1989). Site exposure from a nucleosome internal to a filament is difficult to picture as illustrated in Figure 1, but may be readily imagined as an uncoiling coupled to motion of the uncoiled DNA ends in a direction parallel to the nucleo-

some's DNA superhelix axis. With suitable flexing of linker DNA, such a process allows other nucleosomes in the chain to remain in fixed positions. In any case, recent experiments show that addition of neighbors to a single nucleosome increases rather than decreases the ability of proteins to bind to nucleosomal target sites (Ura *et al.*, 1995) or leads to little qualitative difference in accessibility (Owen-Hughes *et al.*, 1996).

### Site exposure is distinct from nucleosome mobility

A second conclusion from this study is that the detailed mechanism of site exposure (Polach & Widom, 1995, 1996) is distinct from the previously-described process of nucleosome mobility ("sliding"). The actual detailed mechanism of nucleosomal site exposure is not known. The available data are consistent with the hypothesis, but do not prove, that site exposure occurs *via* an uncoiling mechanism as depicted in Figure 1. Indirect evidence arguing against a role for nucleosome sliding in site exposure was discussed previously (Polach & Widom, 1995). However, these new results make this case more directly. Nucleosome mobility occurs on a timescale of tens of minutes to hours (Pennings *et al.*, 1991; Ura *et al.*, 1995), whereas site exposure occurs on a timescale of seconds or faster; thus, the two processes must be distinct. Nucleosome sliding might well result from transcription; but sliding itself occurs too slowly for it to represent the process that makes transcription or regulatory protein binding possible.

We suggest instead that site exposure may contribute as an essential initial step to the mechanism of nucleosome mobility. Perhaps uncoiled DNA segments can be recaptured by the histone octamer at new positions, differing by integral numbers of DNA helical turns from the original contact position, forming a bulged or looped structure as postulated for the case of nucleosome transcription (Studitsky *et al.*, 1995). Propagation of this loop or bulge along the remainder of the nucleosomal DNA would yield a nucleosome that has been displaced from its initial location.

### Catalysis of site exposure *in vivo*

A third and surprising conclusion concerns the actions of RNA and DNA polymerase. Transcription by RNA polII *in vivo* progresses at an average rate of one nucleosome length every  $\approx$  six to ten seconds (Shermoen & O'Farrell, 1992). The slowing down of T7 RNA polymerase on the nucleosomal template observed in this study may be too great to allow the measured natural rate of RNA polymerase II elongation *in vivo*, despite the fact that the T7 polymerase is much smaller than the cellular RNA polymerase II holoenzyme complex and its rate on naked DNA much more rapid. It follows that the progression of RNA (and probably also

DNA) polymerase may need to be catalyzed *in vivo*, either through some property inherent to the eukaryotic polymerases that is lacking in T7 RNA polymerase, or through the recruitment of additional factors to the elongation complex. Factors implicated in the destabilization of nucleosomes are reported to be components of the RNA polymerase II holoenzyme complex *in vivo* (Wilson *et al.*, 1996), although their roles and the mechanism of their action await further study.

We conclude with suggestions for physically plausible mechanisms by which polymerase progression through nucleosomes and/or the rate or extent of nucleosomal site exposure might be catalyzed. (i) A DNA helicase may catalyze site exposure: by allowing the two DNA strands to be released independently, the energy barrier height inhibiting segment release may be reduced. Helicases may be components of polymerase holoenzyme complexes and may be required for action even on naked DNA templates (West, 1996). (ii) A set of mechanisms involves posttranslational modification of histones (e.g. eliminating some of the charge-charge interactions that stabilize histone-DNA interactions). Modifications to the histones could cause increases in rate constants for site exposure, or in equilibrium constants for site exposure, or both. Several enzymes having such activities have recently been purified and cloned (Kleff *et al.*, 1995; Parthun *et al.*, 1996; Taunton *et al.*, 1996; Ogryzko *et al.*, 1996), and many others have been detected.

## Methods

### Reconstitution and characterization of nucleosomal templates

For the transcription studies, the DNA construct used for reconstitution was created by amplification and modification of the 5 S rRNA *Lytechinus variegatus* gene (Simpson & Stafford, 1983) using primers that introduced the promoter for T7 RNA polymerase and the minus-U cassette (Protacio and Widom, 1996). The resulting PCR product was 216 bp long and harbored a 199 nt full-length transcript. The exonuclease III digestion studies use a DNA construct that derives from those used in our analysis of restriction enzyme digestion kinetics (Polach and Widom, 1996), lengthened slightly with the use of different PCR primers. Following purification, trace amounts of the DNA were radiolabeled, mixed with 20  $\mu$ g of unlabeled DNA, and used for reconstitution with histone octamer prepared from chicken erythrocytes (Widom, 1986) at a 1:0.8 DNA to octamer molar ratio in a final volume of 200  $\mu$ l. The reconstitution was performed by a gradual stepwise salt dialysis at 4°C from 2 M NaCl, and successively to 1.5 M NaCl, 1.0 M NaCl, 0.5 M NaCl and finally to low salt conditions (0.5  $\times$  TE, 5 mM NaCl), with each step lasting at least two hours. With the exception of the last step, each buffer was supplemented with

0.5 mM PMSF and 1 mM BZA. After purification by sucrose gradient ultracentrifugation and fractionation, fractions exhibiting expected mobilities for naked DNA or nucleosomes were collected and exchanged into storage buffer (0.5 × TE/5 mM NaCl) using Centricon-30 microconcentrators (Amicon). Approximately 100 ng of naked DNA or nucleosomes were then mixed with loading buffer (3% Ficoll, 5 mM NaCl in 0.5 × TE) and run on a 5% polyacrylamide gel in 1/3 × TBE buffer (30 mM Tris-borate, 0.67 mM EDTA) at 10 V cm<sup>-1</sup>. ImageQuant software (Molecular Dynamics) was used to perform area integration on the phosphorimage of the native gel.

### Transcription reactions

For the analysis of early time-points (Figure 3(a)), each transcription reaction with naked DNA or reconstituted mononucleosomes was carried out in a 200 μl volume. Stalled complexes were formed by mixing the following components in an Eppendorf tube and incubating at 37° for ten minutes: 50 nM template, 1 × transcription buffer (40 mM Tris, pH 8.0; 0.5 mg ml<sup>-1</sup> BSA; 5.0 mM DTT), 25 μM ATP; 25 μM CTP; 225 μM GTP; 1 U μl<sup>-1</sup> RNase-in (Promega); 0.2 mCi ml<sup>-1</sup> [ $\alpha$ -<sup>32</sup>P]ATP (Amersham, 3000 Ci mmol<sup>-1</sup>); 10 mM Mg<sup>2+</sup>; and 1U μl<sup>-1</sup> of T7 RNA polymerase (U.S. Biochemical). After an aliquot was taken for analysis of stalled complexes, the mixture was transferred to a Reacti-Vial Small Reaction Vial (Pierce), which was previously warmed to 37°C. The vial was then placed on a heated stir-plate and insulated with warm anti-freeze. To restart elongation, a Reacti-Vial Magnetic Stirrer (Pierce) was placed in the vial to commence mixing and 10 μl of an NTP mix was added to the reaction with a syringe (Hamilton), bringing the final concentrations of NTPs to 25 μM ATP, 100 μM CTP, 225 μM GTP, and 100 μM UTP. After elongation times of one, two or four seconds, the reaction was then quenched by the rapid addition of 0.5 M EDTA, pH 8.0 with a syringe to bring the final EDTA concentration to 40 mM, followed by the removal of aliquots for the analysis of transcript distribution.

Transcription reactions investigating later time points (Figure 3(b)) were performed in 10 μl volumes using manual pipetting methods. Stalled complexes were formed in conditions identical to the large-volume reactions except that ATP concentration was reduced to 5 μM and [ $\alpha$ -<sup>32</sup>P]ATP was increased to 0.5 mCi ml<sup>-1</sup> (Amersham, 3000 Ci mmol<sup>-1</sup>). To analyze the stalled complexes, a 5 μl aliquot was removed from the reaction and quenched with formamide (94% formamide, 30 mM EDTA, 125 μg ml<sup>-1</sup> xylene cyanol). The stalled complexes were then restarted by the addition of 5 μl of the NTP mix, bringing the final concentration of components to 1 × Transcription Buffer, 10 mM MgCl<sub>2</sub>, 1U μl<sup>-1</sup> RNase-in, 0.25 mCi ml<sup>-1</sup> [ $\alpha$ -<sup>32</sup>P]ATP, 2 mM ATP, 200 μM GTP, 80 μM CTP and 60 μM UTP. After elongation

times of four seconds, one minute, two minutes and five minutes, the reactions were quenched by the rapid addition of 10 μl of the formamide solution. All aliquots were then digested with proteinase K (9.5 μg ml<sup>-1</sup>) at 37° for one hour before analysis on a 6% polyacrylamide/7 M Urea/1 × TBE polyacrylamide sequencing-size gel.

### Exonuclease III digestions

Digestions on DNA and nucleosomes (50 nM total DNA) were carried out in 100 μl reactions in 50 mM Tris-Cl (pH 8.0), 5 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol with 1000 U ml<sup>-1</sup> of Exonuclease III. Digestion was initiated by mixing 50 μl reactions, one containing substrate, the other enzyme, both preheated to 37°C. After the variable reaction times, 10 μl samples were removed and quenched with an equal volume of formamide containing 0.1% xylene cyanol. Each aliquot was then digested with proteinase K (25 μg/ml) at 37°C for two hours prior to electrophoretic analysis (Figure 4).

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