Transcription on nucleosomal templates by RNA polymerase II in vitro: inhibition of elongation with enhancement of sequence-specific pausing

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The process by which RNA polymerase II elongates RNA chains in vivo, where the template is at least partially in a nucleosomal configuration, remains poorly understood. To approach this question we have partially purified RNA polymerase II transcription complexes paused early in elongation. These complexes were then used as substrates for chromatin reconstitution. Elongation of the nascent RNA chains on these nucleosomal templates is severely inhibited relative to elongation on naked DNA templates. Elongation on the nucleosomal templates results in a reproducible template-specific pattern of transcripts generated by RNA polymerase pausing. The RNA polymerases are not terminated because the large majority will resume elongation upon the addition of Sarkosyl or 400 mM KCl. The effectiveness of RNA polymerase II pause/termination sites is enhanced by the presence of nucleosomes. For example, a pause site similar in sequence to the *c-myc* gene exon 1 terminator is used four to seven times more effectively in reconstituted templates. A comparison of elongation on templates bearing phased nucleosomes and on reconstituted templates that show no predominant phasing pattern indicates that the locations of pause sites are not related to the positions of the nucleosomes. Rather, the major determinant of RNA polymerase pausing on the nucleosomal templates appears to be the underlying DNA sequence.

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A number of studies have indicated that transcriptionally active regions may continue to be packaged in nucleosomes while transcription occurs (Nacheva et al. 1989; Chen et al. 1990; Ericsson et al. 1990; Pederson and Morse 1990). This raises the question of how RNA polymerase II can effectively elongate RNA chains on these nucleoprotein templates. Studies with model systems using bacteriophage RNA polymerases and reconstituted templates indicate that nucleosomes need not be insurmountable barriers to RNA synthesis. These single-subunit RNA polymerases can efficiently elongate on both short fragments bearing one or two nucleosomes (Lorch et al. 1987; Losa and Brown 1987; Morse 1989) and on templates with extended arrays of nucleosomes (Pfaffle et al. 1990). RNA polymerase II will efficiently traverse a single nucleosome (Lorch et al. 1987), but the effect of long nucleosomal arrays on polymerase II elongation has not been established.

The necessity for approaching this question biochem-

ically has been emphasized by the growing realization that control of elongation by RNA polymerase II is an important aspect of gene regulation. Premature termination or pausing by RNA polymerase II has been observed in vivo for a number of genes, such as the proto-oncogenes *c-myc* and *c-myb*, the adenosine deaminase gene, and the *hsp70* gene of *Drosophila melanogaster* (for review, see Spencer and Groudine 1990 and references therein). In vitro systems have been employed that reproduce some of the transcriptional pausing/termination events observed in vivo, using naked DNA templates (e.g., see Resnekov and Aloni 1989; Kerppola and Kane 1990). Ultimately, our understanding of these processes will require in vitro systems using both purified transcription factors and templates containing physiological chromatin structure. As an initial exploration of this problem we have developed a system in which paused RNA polymerase II transcription complexes are assembled into chromatin; subsequent elongation by these polymerases can be monitored quantitatively. In this paper we show that elongation is strongly inhibited on

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chromatin templates. The inhibition manifests itself as an inability of the RNA polymerase to traverse certain DNA sequences and is not primarily a function of nucleosome position.

Results

An overview of the experimental approach; the DNA substrates for chromatin reconstitution

Both our laboratory (Knezetic et al. 1988) and other groups (Lorch et al. 1987; Matsui 1987; Workman and Roeder 1987) have shown that assembly of the RNA polymerase II preinitiation complex prior to reconstitution of the template DNA into chromatin allows transcription initiation. The availability of nucleosomal templates bearing initiation-competent RNA polymerases should permit comparison between elongation efficiencies on chromatin and naked DNA templates. However, preinitiation complexes assembled in the manner we had described previously (Knezetic et al. 1988) did not elongate RNA chains effectively over distances of more than several hundred bases. This problem seems to result from the deposition of nonspecific DNA-binding proteins on the template during initiation com-

plex assembly, because the addition of Sarkosyl to the elongation reactions considerably increased the synthesis of longer RNAs (M.G. Izban and D.S. Luse, unpubl.). We showed previously (Knezetic et al. 1988) that transient exposure of templates bearing paused elongation complexes to Sarkosyl followed by gel filtration removes most nonspecific DNA-binding proteins. We found that these Sarkosyl-rinsed templates also efficiently produced very long RNAs on the addition of excess NTPs and Mg^{2+} (e.g., see, Fig. 3, below).

All of the plasmids employed as templates in this study contain adenovirus 2 (Ad 2) major late promoters (see Fig. 1). The pML5 plasmids have a slightly modified downstream region that allows transcription to proceed to +15 in the absence of GTP, thereby forming a paused elongation complex termed 15n complex. Some of the constructs include a single copy (pML5-N) or four copies (pML5-4N and -4NR) of a DNA fragment that has been shown to direct nucleosome assembly to one particular location (Wolffe and Drew 1989).

The procedure for generating the Sarkosyl-rinsed transcription complexes is outlined in Figure 2 (for details, see Materials and methods). Preinitiation complexes were assembled and purified by gel filtration. Transcription was initiated with a subset of the NTPs such that

Figure 1. The vectors pSmaF-1, pML5, and pML5 derivatives. (A) The lightly stippled box represents a *SmaI* fragment from Ad 2, which contains the viral major late promoter. The darkly stippled box (A and B) denotes the major late promoter region (–171 to +35). Restriction sites are indicated: (N) *NruI*; (S) *SmaI*; (D) *DdeI*; (P) *PvuII*. (B) In the pML5-based plasmids, the sequence downstream of the initiation site has been modified to facilitate the production of defined elongation complexes. Positions of the base substitutions are underlined. pML5-N contains a 150-bp fragment (*HindIII*–*PvuII*) of the bacterial CAT gene cloned adjacent to the promoter. This sequence has been reported to specifically position nucleosomes. The DNA predicted to be encompassed within a nucleosome is indicated by the ellipsoid; crosshatching indicates the orientation of the DNA. pML5-4N contains four repeats of the CAT gene, each separated by an identical 30-bp linker. pML5-4NR contains the same tetrameric region cloned in the opposite orientation. Restriction sites are indicated: (B) *BamHI*; (H) *HindIII*; (P) *PvuII*; (S) *SspI*.

Elongation by polymerase II on chromatin templates

Figure 2. Method for preparing partially purified elongation complexes and chromatin templates. The protocols used to prepare the various transcription complexes used in this study are outlined. Note that the column running buffer for the Sarkosyl and salt-rinse steps is the modified chromatin assembly buffer (30 mM Tris-HCl at pH 7.9, 62.5 mM KCl, 10 mM β -glycerophosphate, 0.5 mM EDTA, and 1 mM DTT). Thus, both nonreconstituted and chromatin templates are in identical reaction mixtures for the RNA synthesis step. Because a high surface-to-volume ratio is required to facilitate nucleosome assembly, chromatin reconstitution reactions are performed in small volumes in 1.5-ml microcentrifuge tubes. One batch (190 μ l) of paused elongation complex (15n complex) is sufficient to set up nine chromatin reconstitution reactions. Three of the 40- to 50- μ l assembly reactions are consolidated prior to the salt-rinsing procedure.

discrete populations of paused elongation complexes were generated. These paused elongation complexes were incubated with 1% Sarkosyl and gel-filtered a second time, which removes the Sarkosyl, the proteins released from the DNA by Sarkosyl, and the NTPs. Further elongation of the Sarkosyl-rinsed complexes was performed with cold NTPs. Thus, the amount of radiolabel incorporated into any given transcription product directly reflects the elongation capacity of the RNA polymerase regardless of transcript length.

Elongation on nonreconstituted circular templates

Sarkosyl-rinsed paused elongation complexes, assembled on either pML5 or pSmaF-1, elongated efficiently and relatively synchronously when supplemented with 8 mM MgCl_2 and 600 μM NTPs (Fig. 3). The rate of elongation was ~ 70 nucleotides/min, far less than the estimated 1000 nucleotides/min in vivo elongation rate (Ucker and Yamamoto 1984) but similar to elongation rates observed using partially purified HeLa cell transcription and elongation factors (Reinberg and Roeder 1987). Ammonium ions increase the elongation rate for RNA polymerase II initiated on dC-tailed templates (Sluder et al. 1988). Supplementing the 56 mM KCl normally present in our reactions with 100 mM NH_4^+ increased the elongation rates of complexes on both pML5 and pSmaF-1 to ~ 300 nucleotides/min (Fig. 3). As our Sarkosyl-rinsed templates offer no barriers to elongation except at the known pause sites within pSmaF-1 and at regions similar to the *c-myc* pause/termination signals (Figs. 5 and 7, below), we refer to these templates as naked DNA. The efficiency with which the Sarkosyl-rinsed polymerases traversed the intrinsic pause site at +185 in the pSmaF-1 template is noteworthy, because elongation through this site in vitro requires elongation factors (Rappaport et al. 1987; Reinberg and Roeder 1987) and because there is evidence to suggest that elongation factor TFIIS is removed by Sarkosyl (Hawley and Roeder 1985; Reines et

Figure 3. Elongation on Sarkosyl-rinsed templates. Sarkosyl-rinsed 15n complexes (pML5 template) or Sarkosyl-rinsed complexes generated under NTP-limiting conditions on the pSmaF-1 template were elongated for the number of minutes indicated in modified chromatin assembly buffer after the addition of 8 mM MgCl_2 , 600 μM of NTPs, and NH_4Cl as indicated. The purified transcripts were resolved on a 10% polyacrylamide sequencing gel. The lengths of the DNA size markers run in the right-most lane are indicated at right. The same markers are used in all subsequent figures.

al. 1989). Both the results in Figure 3 and those presented below (e.g., see Fig. 5A) indicate that transcription complexes purified as outlined in Figure 2 are as elongation competent, even during elongation in the presence of Sarkosyl, as any combination of mammalian RNA polymerases and elongation factors that have been reported by other laboratories. Thus, the effects we observe should not be the trivial result of the loss of RNA polymerase II elongation factors.

Elongation on chromatin templates

Sarkosyl-rinsed elongation complexes were assembled into chromatin by overnight incubation in *Xenopus* oocyte extracts essentially as described previously (Knezetic et al. 1988). The extent of reconstitution in selected experiments was monitored by two-dimensional gel electrophoresis of topoisomers (Knezetic and Luse 1986). We routinely generated templates that contained one nucleosome per 190–195 bp (data not shown). Although we have not directly established whether the nucleosomes we assemble are uniformly spaced, we have never observed reconstitutes with significantly higher superhelical densities than those just noted even when we attempted to over-reconstitute with an excess of oocyte extract. This suggests that the nucleosomes we assemble are not tightly packed.

Our initial studies on elongation with chromatin templates utilized 15n complex on pML5 and 12n complex on pSmaF1 as the substrates for chromatin assembly. In contrast to the efficient elongation observed with the nonreconstituted DNA templates (Fig. 3), elongation on the chromatin templates yielded a complex pattern of transcripts with a much shorter average length (Fig. 4, lanes 1, 2, 4, and 5). These shorter transcripts resulted from the accumulation of paused RNA polymerase II elongation complexes (data not shown for this experiment, but see Fig. 8B, below). The inhibition of elongation by chromatin was more pronounced on the pSmaF-1 template than on pML5, particularly at low salt concentrations (Fig. 4, cf. lanes 1 and 4). There was a high level of pausing within the first 200 bp downstream of the promoter, primarily at the pause sites at +120 and +185. These pause sites were also recognized but at a much lower frequency during elongation on naked DNA templates (Fig. 3) and on chromatin templates in the presence of Sarkosyl (Fig. 4, lane 6). The capacity of chromatin to increase pausing at an intrinsic site was also observed with the pML5-N (Fig. 5) and pML5-4NR (Fig. 6) templates.

RNA polymerase pausing can be partially alleviated by adding 100 mM NH_4Cl (Fig. 4, lanes 2 and 5) prior to the addition of NTPs. The effect of NH_4Cl is probably the result of the change in ionic strength and not the result of increasing the rate of elongation, because the addition of either 100 mM KCl or 100 mM NaCl relieves inhibition to a similar extent even though K^+ and Na^+ , unlike NH_4^+ , do not increase the elongation rate of RNA polymerase II on naked DNA (data not shown). The addition of Sarkosyl prior to elongation at a concentration sufficient to disrupt nucleosomes (0.5%) relieved most of the

Figure 4. Elongation on chromatin templates. Sarkosyl-rinsed elongation complexes generated on pML5 (15n complex) and pSmaF-1 (12n complex) were assembled into chromatin and salt-rinsed, as outlined in Fig. 2. The salt-rinsed material was divided into three 50- μl aliquots and supplemented with 8 mM MgCl_2 and with NH_4Cl and Sarkosyl as indicated prior to elongation. Labeled RNAs were purified, as described in Materials and methods, and resolved on 10% sequencing gels. The lengths of the DNA size markers are indicated in the *middle*.

pausing (lanes 3 and 6). The resulting elongation profile was essentially the same as that observed on naked DNA templates. This latter point confirms that the salt-rinsing step used after chromatin assembly does not remove required elongation components. We also verified this directly by chasing the 15n complexes on chromatin templates that were not salt-rinsed; the resulting elongation patterns were identical to those seen on templates rinsed at 250 mM KCl (data not shown).

Elongation on the pML5-N template

We then tested elongation on reconstituted pML-5-based

Figure 5. Elongation on pML5-N chromatin templates; restriction enzyme digestion of 16-hr and 30-min reconstituted templates. Lengths of the DNA size markers for A and B are given at right. (A) Salt-rinsed chromatin templates were assembled as outlined in Fig. 2 and elongated (lanes 2–4) as described in Materials and Methods. Lane 1 contains the elongation products of a Sarkosyl-rinsed template that was incubated for 16 hr at 25°C without addition of the oocyte extract (mock reconstituted) prior to elongation under the Sarkosyl and salt conditions indicated. The ellipsoid marks the nucleosome-positioning sequence. (B) Salt-rinsed pML5-N chromatin templates (150 μ l) (reconstituted either for 16 hr or for 30 min) were digested with 15 units of *Rsa*I for 45 min at 25°C. After an additional salt rinse, which removes the restriction enzyme, the paused 15n complexes were elongated as described in Materials and Methods. The arrowheads mark transcripts extended to the *Rsa*I cleavage sites.

plasmids that contain a single nucleosome positioning sequence downstream of the initiation site (see Fig. 1). Elongation on pML5-N chromatin was greatly inhibited within the predicted nucleosome position (Fig. 5A, lanes 2 and 3). More than half of the RNA polymerases paused while traversing this region under low salt conditions. The effects of NH_4^+ and Sarkosyl on elongation are similar to those observed with pML-5 and pSmaF-1. If Sarkosyl-rinsed templates were simply incubated for 16 hr at 25°C prior to elongation, they exhibited a low degree

of pausing (Fig. 5A, lane 1). The elongation profile was quite different than the pattern generated by chromatin except at the intrinsic pause site at approximately +275. This region of DNA contains a sequence motif similar to known RNA polymerase II pause sites. We will discuss elongation through this class of pause site in more detail below.

To show directly that nucleosomes are responsible for the observed effects on elongation, we took advantage of the relatively slow rate at which nucleosomes are assembled by the oocyte extracts. A 30-min incubation does not lead to nucleosome formation, as judged by the acquisition of superhelical turns, but this time should be sufficient to establish any non-nucleosomal protein–DNA interactions. To demonstrate that nucleosome formation did not occur during the 30-min incubation we also digested the templates with a restriction enzyme, because DNA encompassed within nucleosomes is expected to be less susceptible to restriction cleavage than free DNA (Morse 1989 and references therein). We selected *Rsa*I because it has only two recognition sites in the pML5-N plasmid, one of which occurs within the nucleosome-positioning sequence. After a 45-min digestion, the *Rsa*I was removed by salt-rinsing and the nascent RNAs were elongated. As a control, a chromatin template reconstituted for 16 hr was treated similarly.

The results of this experiment are shown in Figure 5B. The positions of the transcripts that extend to the *Rsa*I cleavage sites are indicated by the arrowheads. We observed very little pausing on templates reconstituted for 30 min, especially when elongation was performed with 100 mM NH_4Cl (lane 5). Approximately 80% of the templates that were briefly exposed to the oocyte extract were digested by *Rsa*I within the nucleosome positioning sequence (+194), indicating that substantial nucleosome formation did not occur. Although we noted a low degree of pausing on the 30-min-incubated pML5-N templates when elongation was performed in 56 mM KCl, nascent RNAs elongated on chromatin templates under the same conditions showed a completely different pausing pattern (lanes 1 and 4). Direct comparison of these lanes, however, should be made with caution. Although lanes 1–3 contain comparable levels of transcription products, the amount of total transcripts in lane 4 is reduced relative to lanes 5 and 6, owing to a decrease in the ability of the 15n complex to restart in low salt after brief exposure to the oocyte extract (for details, see Materials and Methods). As expected, the chromatin templates were much more resistant to *Rsa*I cleavage than the nonreconstituted templates were (only ~20% cleavage per site; see lane 3). The low level of cleavage within the positioning sequence could have a number of explanations: For example, *Rsa*I might be able to cleave within nucleosomes, but this process is inefficient; alternatively, nucleosome assembly might have been less efficient than usual in this experiment. Regardless of the reason, the *Rsa*I-cleaved chromatin retained the chromatin-specific elongation pattern seen on intact templates (cf. Fig. 5A, lanes 2 and 3 with Fig. 5B, lanes 1 and 2). We conclude from all of the results in Figure 5B that the

Figure 6. Elongation on pML5-4N and pML5-4NR chromatin templates and DNase I digestion of pML5-4NR chromatin templates. (A) Elongation was performed as described in Materials and methods with the addition of 100 mM NH₄Cl and Sarkosyl as indicated. (B) Sarkosyl-rinsed 15n complexes, either nonreconstituted (lanes 2 and 4) or reconstituted and salt-rinsed (lanes 3 and 5), were digested for 30 sec with 3 units of DNase I. The DNase was inactivated in both cases by the addition of 0.5% Sarkosyl, and the nascent RNAs were elongated as described in Materials and methods. The purified RNAs were resolved on 6% sequencing gels until the Bromphenol blue (lanes 2 and 3) or the xylene cyanol (lanes 4 and 5) tracking dyes reached the bottom of the gel. Elongation products generated from a portion of the salt-rinsed chromatin (lane 5) prior to DNase I treatment are shown in lane 1. The sizes of the DNA markers are indicated.

pausing we observe on our reconstituted templates is not the result of non-nucleosomal protein–DNA interactions.

Elongation through multimerized nucleosome-positioning sequences

For each of our templates, reconstitution into chromatin and subsequent elongation of the nascent RNAs yielded a discrete, distinctive, and highly reproducible pattern of transcripts. Also, the same transcript patterns were observed when elongation was performed on deliberately under-reconstituted templates. However, the production

of longer RNAs was favored on the under-reconstituted templates, and the average transcript size was inversely proportional to the percent reconstitution (data not shown). We were thus led to the hypothesis that RNA polymerase II has a much higher probability of pausing at certain DNA sequences when these sequences occur at some position within nucleosomes. However, we could not demonstrate that nucleosomes actually do assemble at a single location downstream of the promoter on the pML5-N template. Thus, it was also possible that the specific location of DNA sequences within the nucleosome contributes to the efficiency of elongation through those sites.

To address this issue we constructed a pair of plasmids, pML5-4N and pML5-4NR, each of which contains a tetrameric configuration of the nucleosome phasing sequence in opposite orientations (see Fig. 1). If inhibition of elongation were solely due to the positions of the nucleosomes on the template, irrespective of sequence, then reconstituted pML5-4N and pML5-4NR templates should give the same elongation pattern, except that the bands should be shifted 23 bp downstream for pML5-4NR. However, the elongation patterns on pML5-4N and pML5-4NR chromatin were completely different (cf. Fig. 6A, lanes 1 and 2 with 4 and 5). A strong pause site was observed within each of the repeated sequence units on the reconstituted pML5-4NR template, even when Sarkosyl was present (Fig. 6A, lanes 4–6). This effect was also seen on naked DNA templates (Fig. 7). The pause first occurs at about +195, where the sequence on the nontemplate strand is 5'-TTTTTCTCCATTTT. This is very similar to the sequence (5'-TTTTTGCAGC-ATTTT) at position +275 of the nontemplate strand of

Figure 7. Time course of elongation through naked and nucleosomal templates. Elongation reactions were performed for the times indicated on either naked DNA or reconstituted chromatin by using pML5-4NR template. All reactions were performed at 56 mM KCl. The DNA markers and sizes are shown at right. The DNA predicted to be encompassed within the first nucleosome is indicated by the ellipsoid.

pML5-N, which was also recognized efficiently on naked DNA and chromatin templates (Fig. 5). Both of these sequences are similar to known pause/termination sites that occur within the histone *H3.3* and *c-myc* genes (see Kerppola and Kane 1990).

To demonstrate that nucleosomes occupy specific positions over the chloramphenicol acetyltransferase (CAT) gene sequence on fully reconstituted pML5-4N and pML5-4NR templates we performed DNase I digestion experiments. Digestion of nucleosomal DNA with DNase I results in a diagnostic cleavage pattern with ~10-bp periodicity (see Lutter 1989). Because we were interested in nucleosome positions only on the subset of templates that contained paused transcription complexes, we used the labeled nascent RNA within the 15n complex as the reporter of DNase I cleavage sites. Because RNA polymerase II should terminate at nicks introduced in the coding strand, the length of the labeled RNA generated by elongation on nicked templates would then map the coding strand cleavage sites. It is important to note that the RNA polymerase may be an imperfect reporter of DNase I cleavage sites. We have found that RNA polymerase II often staggers through the final 4–5 bp as it approaches the end of a linear template, resulting in multiple elongation products. Although any given end yields a reproducible set of termination products, we have not found it possible to predict what this set will be (data not shown). Because we did not know how far in advance of any given nick transcription would stop, we could not determine precisely the location of the DNase I cleavage sites. However, because we compared elongation on similarly treated naked and reconstituted templates, we could use differences between these patterns to determine whether nucleosomes occupied one or several specific positions within the CAT sequences of pML5-4NR.

We digested pML5-4NR naked DNA (Fig. 6B, lanes 2 and 4) and salt-rinsed chromatin (lanes 3 and 5) templates with DNase I. DNase I activity was terminated (and the nucleosomes were dissociated from the chromatin template) by adding Sarkosyl prior to elongation. The first nucleosome on pML5-4NR is expected to extend from +87 to +237 (see Fig. 1). Consistent with this prediction, there were major differences in the digestion patterns of naked DNA and nucleosomal templates in this region. Chromatin templates were hypersensitive to DNase I cleavage at position +81 (Fig. 6B, cf. lanes 2 and 3), which approximates the predicted DNA entry point into the first nucleosome. Downstream of this point the DNase I sensitivity of the chromatin template rose and fell periodically relative to that of the naked DNA. This effect was stronger within the first half of the nucleosome-positioning sequence, and the DNase I sensitivity in the region surrounding the presumptive midpoint or dyad axis of the nucleosome (+164) was reduced. This overall digestion pattern was also observed with pML5-4N (data not shown). The suppression of cleavage within and downstream of the region of dyad is consistent with previous reports of DNase I digestion patterns using bulk measurements of native chromatin (Lutter 1989) and on

in situ measurements of SV40 minichromosomes (Zhang and Gralla 1989).

We did not generate the completely regular 10- to 11-bp DNase I pattern over the nucleosome-positioning sequence that was observed with short linear end-labeled DNA fragments bearing this positioning sequence reconstituted using the salt-urea dialysis method (Wolffe and Drew 1989). As noted above, our methods might not generate such a result even if the nucleosomes assembled at only one position. It is also possible that nucleosomes assembled in a limited number of different phases on pML-4NR. However, we are confident that nucleosomes did not assemble randomly or in a large number of alternative phases in the experiment shown in Figure 6, because in either of these cases the nuclease digestion pattern on the chromatin templates would have been indistinguishable from that obtained with free DNA templates. We cannot imagine a case in which more than several superimposed alternative phases could produce the long stretches of essentially complete nuclease protection (e.g., the region between +119 and +137) that we observed. In any event, regardless of whether the nucleosomes occupy one or a small number of defined positions on reconstituted pML5-4NR templates, the conclusion that we draw from the comparison of the elongation patterns on nucleosomal pML5-4N and pML5-4NR templates (Fig. 6A) remains the same; namely, that underlying DNA sequence and not nucleosome position is the major determinant of pause sites on chromatin templates. One other set of observations also supports this idea. We were not able to reproduce the positioning shown in Figure 6B in every experiment. In some cases, the DNase I digestion pattern for the reconstituted sample was only slightly different from that obtained with the nonreconstituted sample, even though chromatin assembly as judged by supercoiling was as complete as for the sample shown in Figure 6B. However, the pattern of transcriptional pausing seen on these templates was identical to that observed when nucleosomes predominantly assembled in the expected location, for both pML5-4NR and pML5-4N (data not shown).

To investigate the effects of nucleosomal templates on the subsequent rate of elongation by RNA polymerase II, we performed an elongation time course on naked and reconstituted pML5-4NR. Consistent with the data in Figure 3, polymerases elongating in 56 mM KCl moved relatively synchronously at ~70 nucleotides/min on naked pML5-4NR DNA templates (Fig. 7). The large majority of RNA polymerases efficiently traverse the intrinsic pause site at +195 on naked DNA templates (see the 3- to 5-min elongations; additional data not shown). However, the polymerases that stop at the intrinsic pause site appear to be trapped because the amount of transcript at this site was not significantly reduced upon further incubation. We do not know whether the complexes that produced these transcripts have paused but are capable of restarting. Alternatively, they may have terminated and released their nascent RNAs or they may have become stable, elongation-incompetent ternary

complexes, analogous to the dead-end complexes described by Arndt and Chamberlin (1990) with *Escherichia coli* RNA polymerase.

The elongation rate on nucleosomal templates appeared to be similar to that observed on naked DNA over the region between the paused 15n complex and the first nucleosome-positioning sequence (cf. naked and chromatin 0.5- and 1.0-min time points). However, paused elongation complexes accumulated during transcription throughout the region of DNA encompassed within the first nucleosome (indicated by the ellipsoid). Elongation complex movement on the chromatin templates was essentially complete after 3–4 min of incubation, although a small fraction of the polymerases elongated further by 10 min. Extending the incubation to 20 min resulted in very little additional elongation.

Elongation as a function of salt concentration; can the elongation intermediates paused on chromatin templates be restarted?

As noted above, the elongation potential of RNA polymerase II on chromatin templates is increased at higher ionic strength. To study this point systematically we monitored elongation efficiencies at KCl concentrations in the 56–400 mM range with the pML5-4N and pML5-4NR templates. Increasing the salt concentration (Fig. 8A) relieved the inhibition of elongation; most of the pausing was alleviated between 250 and 400 mM KCl. We obtained a similar result when pML5 and pSmaF-1 were used as templates and when NaCl was substituted for KCl (data not shown). Although salt concentrations of 400 mM greatly reduced the inhibition of elongation, salt rinsing with salt concentrations as high as 670 mM had no effect on the subsequent pausing pattern when elongation was carried out at lower salt concentrations (data not shown). The fact that elongation was enhanced at higher salt concentrations is consistent with the notion that the stability of the nucleosome results from the sum of many histone–DNA contacts along the entire core particle. Even though nucleosomes do not dissociate from the DNA at 400 mM KCl, elongation under these conditions is presumably enhanced by destabilization of the individual DNA–histone interactions.

We also investigated whether the RNAs produced on reconstituted templates were retained in elongation-competent ternary complexes. We divided salt-rinsed reconstitutes made with either the pML5-4N or pML5-4NR template into three reactions and performed initial elongations for 10 min in 56 mM KCl. As a control, elongation was continued for 10 min in one of the reactions without the addition of KCl or Sarkosyl. The other two reactions were supplemented with either KCl or Sarkosyl followed by an additional 10-min elongation. If the transcription complexes remained elongation competent, then the subsequent addition of KCl or Sarkosyl should disrupt histone–DNA interactions and enable elongation to proceed. Both KCl and Sarkosyl were able to release the majority of the RNA polymerases paused during the first 10 min elongation on pML5-4N and

Figure 8. Elongation through chromatin as a function of KCl concentration and pausing vs. termination analyses. Elongation reactions were performed on reconstituted chromatin by using the indicated DNA templates. Sizes of the DNA markers are given at *left* and *right*. (A) Salt-rinsed chromatin (200 μ l) was collected, and 40- μ l aliquots were elongated at the indicated KCl concentrations; Sarkosyl was added as indicated. (B) Elongation reactions were performed on 50- μ l aliquots in 56 mM KCl for 20 min (lanes 1 and 4) or for 10 min (lanes 2, 3, 5, and 6) prior to supplementation with KCl or Sarkosyl as indicated, followed by an additional 10-min incubation.

pML5-4NR (cf. Fig. 8B, lane 1 with lanes 2 and 3, and lane 4 with lanes 5 and 6). The resulting elongation profiles on either template were similar to those produced when KCl or Sarkosyl was added prior to elongation (Fig. 8A).

Quantitation of elongation efficiency on the pML5-4NR template

It is clear that packaging the DNA template in nucleosomes considerably reduces the efficiency of elongation by RNA polymerase II. To simplify the problem of expressing this change quantitatively, we first determined the effect of chromatin on elongation through intrinsic pause sites in pML5-4NR; these sites are recognized on naked DNA templates at measurable levels. As noted above, polymerases that stop at these sites are essentially irreversibly paused. For the sake of simplicity we refer to the percent of polymerases that remain at the pause site as the pausing frequency, even though some proportion of the polymerases may actually have terminated transcription. Pausing frequencies were calculated by determining the number of counts at a particular site and dividing this value by the number of counts at and

above that site by using the set of reactions shown in Figure 9 (for details, see Materials and methods).

The pausing frequency was 12% at the first two pause sites on naked DNA templates in the low salt (56 mM KCl) reaction condition (lane 4). The addition of 100 mM NH_4Cl (high salt) decreased pausing at the first two sites roughly twofold, to $\sim$ 5% (lane 5). We could not determine pause frequencies accurately at the third and fourth sites because of poor resolution, but the estimated percentages were similar to those calculated for the other sites. The ability of the RNA polymerase to pass through these pause sites in chromatin was dramatically decreased. The low and high salt pausing frequencies were $\sim$ 48% and 37%, respectively. Therefore, the overall pausing frequency increased either four- or sevenfold on chromatin templates, depending on the salt concentration. These results provide a quantitative indication of the extent to which the presence of nucleosomes alters the efficiency at which RNA polymerase II elongates through certain pause sequences. Finally, the addition of Sarkosyl and high salt prior to elongation, which should remove nucleosomes from the template, reduced the pausing frequencies to $\sim$ 10%, which is similar to the naked DNA intrinsic pause frequency (lane 3).

Figure 9. Quantitation of elongation capacities on nonreconstituted and reconstituted pML5-4NR template. Elongation reactions were carried out, and the fraction of the RNA polymerases paused at or before the major intrinsic pause sites (asterisks) and within the nucleosome-positioning sequence were determined as described in Materials and methods. Sarkosyl and/or 100 mM NH_4Cl were added to the elongation reactions as indicated. Sizes of the DNA markers are given at right.

The cumulative effect of increased pausing at many nonintrinsic sites on pML5-4NR chromatin produces a dramatic difference in overall elongation efficiency relative to naked DNA templates. On naked DNA only 3% of the total transcripts failed to reach the first intrinsic pause in either low salt or high salt conditions. In chromatin, however, 73% and 47% of the total transcripts were present below the first intrinsic pause site in low and high salt, respectively. In the high salt reaction >65% of the RNA polymerases were paused within the first nucleosome positioning sequence on pML5-4NR.

Discussion

We find that the efficiency of elongation on nucleosomal templates is greatly reduced relative to elongation on

nonreconstituted controls. This reduction in efficiency is due to an increase in RNA polymerase pausing. At first glance, our results may seem to contradict an earlier study that reported efficient elongation by RNA polymerase II over short, linear templates bearing a single nucleosome (Lorch et al. 1987). However, our assessment of reduced elongation efficiency is based, first, on the relative inability of nucleosomal templates to support synthesis of long (>1000 nucleotides) RNAs, and second, on the manyfold increase in pausing at certain sequences that are recognized as pause sites even on naked DNA templates. Our data indicate that nucleosomes are not insurmountable barriers to elongation, particularly at physiological salt concentrations (Fig. 8A), in contrast to recent results with *lac* repressor (Deuschle et al. 1990) and perhaps a CCAAT box-binding protein (Connelly and Manley 1989). The findings reported here are similar to those recently obtained for RNA polymerase III. It has been demonstrated that elongation by polymerase III is inhibited on templates containing long arrays of nucleosomes. However, elongation was more efficient on templates where the transcribed region contained only one to four nucleosomes (Felts et al. 1990). Morse utilized restriction enzyme digestion of minimally reconstituted chromatin templates to show directly that the elongation potential of RNA polymerase III is inhibited by a single nucleosome via a "premature termination" mechanism (Morse 1989).

In contrast to the eukaryotic RNA polymerases, bacteriophage RNA polymerases will efficiently elongate on nucleosomal templates (Lorch et al. 1987; Losa and Brown 1987; Morse 1989; Pfaffle et al. 1990). The reasons for this difference are not clear, but it is worth noting that bacteriophage RNA polymerases are capable of multiple rounds of initiation per template. Decreasing the concentration of the phage polymerase, which probably reduces the number of initiation events per template, decreases elongation efficiency through nucleosomes (Losa and Brown 1987; Morse 1989). Our experiments are designed so that each active template can bear only a single transcription complex. Therefore, we cannot test the possibility that effective elongation on chromatin templates requires multiple RNA polymerases transcribing the same template.

Perhaps our most striking observation is that inhibition of elongation over nucleosomal templates results from increased pausing by RNA polymerase II at particular DNA sequences. These paused complexes, perhaps with the exception of those paused at the intrinsic pause site of pML5-4NR, are fairly stable and the majority of them are released upon subsequent addition of Sarkosyl or high levels of KCl (Fig. 8B). Thus, RNA polymerase II pausing on nucleosomal templates does not drastically alter the elongation competence of the stalled complex. Our plasmids contain several intrinsic pause sites (particularly the +275 stop in pML5-N and the +195 stop in pML5-4NR) that are very similar to known RNA polymerase II pause sites in the *c-myc* and histone *H3.3* genes (see Kerppola and Kane 1990; Spencer and Groudine 1990). Pausing at the pML5-4NR site increased sev-

enfold (150 mM salt) and fourfold (56 mM salt) on nucleosomal templates and returned to nearly the naked DNA level when Sarkosyl was introduced prior to elongation. This is the first direct demonstration that the efficiency of elongation by RNA polymerase II through a particular site can be altered by chromatin structure. It is interesting that although the addition of 100 mM NH_4Cl decreases pausing on both naked and chromatin pML5-4NR templates, the extent of reduction is lower for chromatin. Thus, the fold increase in pausing at the major 4NR pause site is actually greater at the higher salt concentration. This emphasizes the importance of using DNA in a chromatin configuration to reliably assess the behavior of biologically important pause sites *in vitro*.

We have performed preliminary tests on the rate of elongation of RNA polymerase II on naked and reconstituted pML5-4NR templates (Fig. 7). We found that elongation rates were identical over the first 80 bases, where nucleosomes are not expected to occur on the assembled templates. This underscores our conclusion that nucleosomes are the source of the inhibition of RNA chain extension. Most interestingly, when the transcript patterns generated over the next 100 bases were compared, many of the pause sites recognized on chromatin were also found transiently in the naked DNA elongation pattern. However, the chromatin pattern was not simply a copy of the transient naked DNA pattern; some bands were unique to one reaction or the other, and many common bands appeared at considerably different intensities in the two patterns (Fig. 7; additional data not shown). If these results prove to be general they will indicate that the inhibition of elongation by nucleosomes is not simply due to a uniform increase in the residence time of the RNA polymerase at all pause sites. We have not yet tested our Sarkosyl washing and chromatin assembly protocols on templates other than those described here. Thus, we cannot comment on whether any of the effects we observe are promoter dependent. Differential promoter usage may be involved, at least in part, in the control of termination at the *c-myc* pause/termination signal located at the 3' end of the first exon (Spencer et al. 1990).

In summary, we have demonstrated using an *in vitro* system that elongation by RNA polymerase II is inefficient on nucleosomal templates. Transcriptional pausing is strongly enhanced, particularly at sites that resemble intragenic sites known to control elongation *in vivo*. Our results emphasize the importance of considering the control of elongation by RNA polymerase II in the context of chromatin structure. The experimental approach we describe here should prove useful in assaying the effect on elongation of both transcription factors and modifications of chromosomal proteins.

Materials and methods

Plasmid construction

pSmaF-1 contains 2450 bp of Ad 2 DNA bearing the major late promoter cloned into pBR322 (Knezetic and Luse 1986). The

construction of pML5 is described in detail elsewhere (G.A. Jacob, S.W. Luse, and D.S. Luse, *in prep.*). Briefly, a 209-bp fragment containing a modified Ad 2 major late promoter was cloned into the *Sma*I site of pUC18 such that transcription is directed toward the *Hind*III site. The base changes important for the work reported here are (on the noncoding strand) a C-to-G change at +11 and a T-to-A change at +13 (see Fig. 1). Thus, ApC-primed transcription of pML5, using only UTP and CTP as substrates, leads to the synthesis of a 15-nucleotide RNA.

pML5-N was constructed by removing a 150-bp *Hind*III–*Pvu*II fragment of the CAT gene expected to contain a nucleosome-positioning sequence from pRSVcat (Wolffe and Drew 1989) and cloning it into pML5, which had been cleaved at the unique *Hind*III and *Ssp*I sites. To multimerize the *Hind*III–*Pvu*II portion of the CAT gene we first cloned this DNA segment into *Hind*III–*Sma*I-digested pUC18. The resulting construct (pUC-N) contains an *Xba*I site adjacent to the *Pvu*II–*Sma*I junction. We then isolated the *Xba*I–*Hind*III fragment from pUC-N and ligated the following linkers onto it:

AGCTTACGCTGCAGCAACGGA and CTAGACATGCTCTGCCC	
ATGCGACGTCGTTGCCT	TGTACGACACGGG
(<i>Hind</i> III/blunt)	(<i>Xba</i> I/blunt)

The sizes of the linkers were chosen to guarantee that monomers of the nucleosome-positioning signal would be long enough to accommodate both a nucleosome and a physiological length spacer. The *Xba*I–*Hind*III pUC-N fragment with the attached linkers was cloned into *Xba*I–*Pst*I-digested and blunt-ended pML5. The resulting clone (pML5-1N) contained the CAT insert in an orientation similar to pML5-N. pML5-1N was digested with *Bam*HI (located 5' to the CAT gene insert), blunt-ended, and digested with *Sph*I (located 3' to the CAT insert). This fragment was cloned into pML5-1N that had been digested with *Xba*I, blunt-ended, and subsequently digested with *Sph*I. Because both *Xba*I and *Sph*I are 3' of the CAT insert, this construction resulted in a direct repeat of the insert. We call this dimeric construction pML5-2N. pML5-4N was generated by essentially repeating the *Bam*HI–*Sph*I and *Xba*I–*Sph*I digests used for pML5-2N, except that a partial *Bam*HI digestion was necessary because the ligation of the blunt-ended *Bam*HI and *Xba*I sites regenerated the *Bam*HI site. pML5-4NR was made by partially digesting pML5-4N with *Hind*III prior to digesting with *Sph*I. The large *Hind*III–*Sph*I fragment containing all four nucleosome-positioning sequences was isolated and cloned into pML5 that had been linearized with *Sph*I and *Hind*III. All of the constructions were verified by sequencing before use.

Assembly of transcription complexes; elongation reactions

RNA polymerase II preinitiation complexes were formed on Ad 2 major late promoter-bearing plasmids as described previously [Luse et al. 1987], except that the reaction volume was reduced to 150 μ l, the concentration of DNA was 19 μ g/ml, and the protein–DNA complexes were isolated by gel filtration on 1.65 ml (0.5 $\times$ 8.5 cm) Bio-Gel A-1.5m (Bio-Rad) columns. Because DNA–protein complexes are excluded from the resin, the majority of the void volume (~160 μ l) was collected. The Bio-Gel-purified preinitiation complexes were used directly for subsequent procedures.

Stable elongation complexes paused at defined positions were generated on the pML5 derivatives by incubating 100 μ l of the gel-filtered preinitiation complex in a 125- μ l reaction containing 2 mM ApC (Sigma), 10 μ M UTP, 10 μ M dATP, and 0.5 μ M [α - 32 P]CTP (800 Ci/mmol, NEN). All nonradioactive nucleotides were from Pharmacia (FPLC purified). After 5 min at

25°C, the reactions were chased with 10 μ M UTP and CTP. This results in the large majority of the elongation complexes pausing after the incorporation of CTP at +15, just prior to the addition of the first G residue (see Fig. 1). Because a low level of GTP contamination is unavoidable, all elongation complex preparations also contained a small proportion of complexes paused at +18, prior to the G residue at position +19. For the sake of simplicity we refer to this population of paused elongation complexes as 15n complex. For the Ad 2 major late promoter in pSmaF-1, the substrates used were either 50 μ M ATP, 50 μ M CTP, 1 μ M GTP, and 0.15 μ M [α -³²P]UTP (Fig. 3) or 2 mM ApC, 10 μ M UTP, 10 μ M dATP, 1 μ M GTP, and 0.5 μ M [α -³²P]-CTP (Fig. 4). The former condition creates a series of elongation complexes paused at positions 7, 10, 13, and 17, prior to the incorporation of UTP (Coppola and Luse 1984). The latter approach yields predominantly a 12n complex paused prior to incorporation of the first A residue at +13.

To generate purified elongation complexes, gel filtration was again employed, in this case with the addition of Sarkosyl (to which the elongation complex, but not the preinitiation complex, is resistant; Cai and Luse 1987). The elongation reactions were brought to 1% Sarkosyl and incubated for 5 min at 25°C prior to Bio-Gel A-1.5m chromatography. Columns of 1.65 ml were used as before, but the running buffer during this step was a modified version of the MEM chromatin reconstitution buffer (MEM) where 0.5 mM EDTA replaces 2 mM EGTA (30 mM Tris-HCl at pH 7.9, 10 mM β -glycerophosphate, 62 mM KCl, 0.5 mM EDTA, and 1 mM DTT). Before the reaction mixture was loaded, 130 μ l of 1% Sarkosyl in MEM was applied to each column. Thus, the transcription complexes pass through a zone of 1% Sarkosyl within the column, allowing proteins removed from the DNA to be fractionated away from the transcription complex before the complexes themselves are separated from the detergent. We refer to this procedure as Sarkosyl rinsing. Nearly 80% of the input DNA and only ~6% of the initial protein were recovered in the 190 μ l of void volume collected from the second gel filtration step (data not shown).

Elongation reactions using Sarkosyl-rinsed templates were performed by simply adding MgCl₂ and NTPs to 6 mM and 800 μ M, respectively, followed by incubation at 30°C for 10–15 min. Greater than 80% of the paused polymerases remained fully elongation competent. The reactions were terminated by adding EDTA to a fourfold excess over Mg²⁺, Sarkosyl (0.5%) to the reactions that did not contain it and proteinase K (100 μ g/ml). Proteinase digestion was performed at 37°C for 1.75 hr, followed by extraction with phenol/CHCl₃ (1:1) and CHCl₃ alone. RNAs were precipitated with EtOH, washed with 70% EtOH, and resuspended in 2 μ l of RNase-free TE (10 mM Tris at pH 8.0 and 1.0 mM EDTA). Formamide dye mix was added (4 μ l), and the samples were boiled for 3 min, quick-chilled on ice, and electrophoresed on 10% polyacrylamide sequencing gels (29:1 acrylamide/bis) containing 7.8 M urea. After electrophoresis the gels were exposed (usually for 18 hr) to Kodak X-AR film with a Lightning Plus intensifying screen.

Elongation efficiencies were quantitated by using the Phosphorimager system from Molecular Dynamics (Sunnyvale, CA). The intensity of the computer image generated by this instrument for a particular region of a gel is directly proportional to the radioactivity within that region over a much greater range of counts than we employed in our studies. Because all transcripts are uniformly labeled within the first 15 nucleotides, the fraction of RNA polymerases that elongate to any given length can be calculated by determining the number of pixels in the band or region in question divided by the total number of pixels present in all the transcription products. Pausing frequencies were measured by determining the number of pixels at the tran-

script size of interest and dividing by the total number of pixels at and above that transcript.

Chromatin assembly

Xenopus oocyte extracts were prepared as described (Glikin et al. 1984). Reconstitution was performed in a 1.5-ml microcentrifuge tube for 16 hr at 25°C by using 20 μ l of Sarkosyl-rinsed elongation complexes (~150 ng of DNA) and 20–35 μ l of oocyte extract that was made 0.5 mM EDTA. The usual batch of paused elongation complex (~190 μ l) was divided into smaller aliquots for reconstitution because a high surface-to-volume ratio is required to facilitate nucleosome assembly (Glikin et al. 1984). Each preparation of extract was titrated to determine the amount of oocyte extract required to fully reconstitute plasmid DNA, as judged by reacquisition of superhelical tension in the plasmid (for a complete description of this assay, see Knezetic and Luse 1986). To determine the exact topoisomer distribution of the reconstituted template, two-dimensional gel electrophoresis was performed as described (Knezetic and Luse 1986).

Elongation on chromatin templates

To remove nonhistone DNA-binding proteins from the reconstitutes, chromatin was routinely salt-rinsed in 250 mM KCl before elongation of the nascent RNAs. This is the analogous procedure to Sarkosyl rinsing, except that 250 mM KCl is substituted for 1% Sarkosyl. Three 50- μ l chromatin reconstitution reactions were pooled, and 10.2 μ l of 3 M KCl was added; after a 10-min incubation the mixture was gel-filtered at room temperature on a 0.5 $\times$ 8.5-cm Bio-Gel A-1.5m column. The running buffer was MEM, and each column was preloaded with 130 μ l of MEM containing 250 mM KCl. Six 40- μ l fractions were collected beginning at the leading edge of the void volume (~480 μ l). The four fractions with the highest level of nascent RNA, judged by Geiger monitor, were pooled. The remaining fractions were used to assay the extent of reconstitution as described (Knezetic and Luse 1986). Although there was a significant release of nascent RNA during incubation in the oocyte extract and subsequent column chromatography, ~80% of the surviving complexes were fully elongation competent.

Each 160- μ l pool of salt-rinsed reconstitute was typically divided into three tubes of 50 μ l each. Transcripts were elongated by adding MgCl₂ to 8 mM and NTPs to 800 μ M. The final KCl concentration in the nonsupplemented reaction was 56 mM. Elongation was performed with or without the addition of NH₄Cl to 100 mM and Sarkosyl to 0.5%, as indicated in the figures. Elongations were run at 30°C for 10–15 min. RNAs were purified and resolved by polyacrylamide gel electrophoresis as described above.

DNase I digestions

DNase I digestions were performed on 150 μ l of salt-rinsed chromatin by adding 2.1 μ l of 0.5 M MgCl₂, equilibrating the reactions to 25°C, and adding 3 μ l of RNase-free DNase I (RQ-1, 1 U/ μ l; Promega). The chromatin was digested for 30 sec, and the reaction was stopped by adding 8 μ l of 10% Sarkosyl. The nascent RNAs were then elongated for 15 min at 30°C after the addition of 15 μ l of 10 mM NTPs. The reactions were stopped by adding 5 μ l of 0.5 M EDTA (pH 8.0) and digested with proteinase K (100 μ g/ml) for 45 min at 37°C. The RNA was purified and prepared for electrophoresis as described above; the samples were run on a 6% gel with a 19:1 acrylamide/bis ratio. The short and long runs were electrophoresed until either the Brom-

phenol blue or the xylene cyanol dye marker reached the bottom. After electrophoresis the gels were transferred from the glass plate to Whatman 3MM filter paper, dried, and autoradiographed as described above. DNase I digestions of naked DNA were performed as described for chromatin, except the starting material consisted of 60 μ l of Sarkosyl-rinsed DNA brought up to 150 μ l by adding 90 μ l of MMEM.

RsaI digestions

RsaI digestions were performed on 150 μ l of salt-rinsed chromatin that was assembled for either 16 hr or 30 min by adding 2.1 μ l of 0.5 M $MgCl_2$ and 1.5 μ l of 15 U/ μ l of *RsaI* (New England Biolabs). Digestion was performed at 25°C for 45 min. The reactions were salt-rinsed again as described above, except that the reaction mixture was loaded onto the Bio-Gel column immediately after the KCl addition. The nascent RNAs were then elongated, purified, and resolved by electrophoresis as described above. Quantitation of the percent *RsaI* cleavage was performed using the PhosphorImager system as described above.

The capacity of the paused elongation complex to restart in 56 mM KCl differs depending on the reconstitution time and the number of salt rinses performed. For instance, the capacity of the paused polymerases to restart in 56 mM KCl is virtually abolished after brief exposure to the oocyte extract followed by a single salt rinse. This inhibition is greatly relieved after two salt rinses (Fig. 7, lane 4). The inhibition is fully relieved when elongation is performed in 100 mM NH_4Cl regardless of the number of salt rinses (Fig. 7, lane 5). On the other hand, the capacity of polymerase to restart in 56 mM KCl after an overnight assembly, whether or not the chromatin was salt-rinsed, is not altered even when elongation is performed at 56 mM KCl (data not shown).

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