Nucleic Acids Research, 1998, Vol. 26, No. 4 **1051–1055**

# Nucleosomes are not necessary for promoter-proximal pausing *in vitro* on the *Drosophila hsp70* promoter

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Received September 29, 1997; Revised and Accepted December 11, 1997

**RNA polymerase II has been found to pause stably on several metazoan genes in a promoter-proximal region located 20–40 nt downstream from the start site of transcription. Escape of polymerase from this paused state has been proposed to be a rate limiting step in transcription of some genes. A study of the human *hsp70* promoter showed that a nucleosome positioned downstream from the transcription start was a key component in establishing a stably paused polymerase in one cell-free system. We tested whether these results could be extended to the *Drosophila hsp70* promoter in a *Drosophila* cell-free system and found that polymerase paused stably on the promoter even when the length of DNA downstream from the transcription start was not sufficient for assembly of a nucleosome. Our results indicate that a downstream nucleosome is not a universal requirement for stably pausing RNA polymerase in the promoter-proximal region.**

## INTRODUCTION

Analysis of several promoters in mammalian and *Drosophila* cells has revealed the presence of polymerase molecules that are paused on the DNA template in the region 20–40 nt downstream of the transcription start (1–4). Previous studies have suggested that regulation of this pausing could be a key point for controlling gene expression. For example, paused polymerase is evident on the c-*fos* promoter in quiescent cells (3). This state could be due to a repressive mechanism that keeps the gene from being expressed or it might represent a potentiated state that allows for rapid activation of the c-*fos* gene if cells receive a signal to resume the cell cycle. A similar situation exists for the *hsp70* promoter of *Drosophila* (4 and references therein). In this case the paused polymerase is evident when cells are under non-heat shock conditions, during which the gene is not expressed. When both of these promoters are induced there is evidence that pausing still occurs. Hence, pausing could be a rate limiting step of transcription whether the gene is inactive or active.

The mechanism for promoter-proximal pausing is not known. Recent evidence suggests that a nucleosome might be involved in pausing polymerase near the transcription start site. To reconstitute a stably paused polymerase on the human *hsp70* promoter, Brown *et al*. (5) found that they had to assemble the DNA template into chromatin. For this reconstitution RNA polymerase was initiated on an immobilized template in a HeLa cell extract and forced to pause at +15 by withholding a nucleotide. The elongation complex was then washed with sarkosyl, which is expected to strip most proteins from the template while leaving the elongation complex intact. In the absence of nucleosomes, addition of nucleotide triphosphates allowed the polymerase to resume elongation. A transient pause, lasting <10 min was observed. However, if the elongation complexes were incubated in a chromatin assembly mixture consisting of a *Xenopus* extract and core histones, polymerase was observed to pause in regions around 20 and 40 nt downstream of the transcription start. The pause that occurred in the context of reconstituted chromatin lasted for at least 6 h.

Because of the results obtained for the human *hsp70* promoter, we were interested in determining if a nucleosome was required for polymerase to pause on the *hsp70* promoter of *Drosophila*. Pausing on the *hsp70* promoter of *Drosophila* has been extensively analyzed *in vivo* and in isolated nuclei (4 and references therein). Under non-heat shock conditions when *hsp70* is not transcribed, RNA polymerase molecules are observed to be paused in the region 20–40 nt downstream of the transcription start. We recently demonstrated that promoter-proximal pausing can be reconstituted on the *Drosophila hsp70* promoter in nuclear extracts from non-heat shocked *Drosophila* embryos (6). This paused polymerase was detected by treating the DNA with $KMnO_4$ and analyzing the pattern of oxidation that occurred. Thymine residues located in the transcription bubble of the paused polymerase react highly with permanganate. We showed that the pattern of permanganate reactivity observed *in vitro* was strikingly similar to the pattern observed in isolated nuclei and have recently shown that the same pattern of permanganate reactivity is observed in intact salivary gland cells (7). Here we use this cell-free system to investigate the role of nucleosomes in pausing on the *Drosophila hsp70* promoter.

## MATERIALS AND METHODS

### Materials

Nuclear extract was prepared from 0–12 h *Drosophila* embryos as described by Biggin and Tjian (8). pUC.XBS.*hsp70*(–194/+84) and pUC.XBS.*hsp70*(–194/+42) were constructed by Emanuel and Gilmour (9) and purified by CsCl gradient centrifugation. The plasmids were digested with *Hin*dIII and analyzed on an agarose

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gel to confirm complete digestion (data not shown). The digested plasmids were partially protected from degradation by nucleases present in the nuclear extract by incorporation of dATPαS (Fig. 1) or a dNTPαS mixture (Fig. 2) by Klenow fragment (10). To analyze the permanganate reactivity of these templates, a primer (5′-TGG GCT GCA GGT CGA CC-3′) complementary to sequences between the *Hin*dIII site and the site of *hsp70* insertion (see Fig. 1A) was synthesized, called PL1. The primer was labeled with [$\gamma$-$^{32}$P]ATP by T4 polynucleotide kinase (US Biochemical) and separated from unincorporated nucleotides with a Nensorb 20 column according to the manufacturer's protocol.

### $KMnO_4$ analysis of pausing *in vitro*

*In vitro* transcription reactions (40 µl) were set up containing 30% *Drosophila* nuclear extract, 10 ng template DNA, 1 µg *Hae*III-digested *Escherichia coli* DNA, 32.5 mM HEPES–K, pH 7.6, 0.8 mM Tris–HCl, 0.13 mM EDTA, 6.25 mM $MgCl_2$, 50 mM KCl, 5% glycerol and 0.5 mM DTT (all concentrations final). Some reactions contained 5 µg/ml α-amanitin and/or 0.3 mM NTPs, as indicated. After a 30 min incubation at 21°C, 5 µl 0.3 M $KMnO_4$ were added and the sample was incubated at 21°C for 4 min. The reactions were stopped with 5 vol. of a solution containing 50 mM EDTA, pH 8.0, 1% SDS and 0.4 M 2-mercaptoethanol. The mixture was treated with proteinase K, extracted with phenol/chloroform/isoamyl alcohol (24:24:1), precipitated with ethanol and dissolved in water. The Taq extension analysis was performed with primer PL1 using essentially the protocol described by Li *et al*. (6). After 25 cycles of primer extension the samples were extracted with phenol/chloroform/isoamyl alcohol (24:24:1), precipitated with ethanol, dissolved in gel loading buffer (98% formamide, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue, 10 mM EDTA, pH 8.0) and analyzed on a 7 M urea, 8% polyacrylamide gel.

### Analysis of *in vitro* transcripts

*In vitro* transcription reactions (40 µl) were set up as described above in the absence of NTPs to allow transcription preinitiation complexes to form. Transcription was initiated by addition of NTPs to 0.3 mM. At the times indicated in Figure 2, 5 µg/ml α-amanitin (or water) were added. Six and 30 min after NTP addition, 15 µl of the reaction were removed and added to 55 µl solutions containing 8 mM HEPES–K, pH 7.6, 16 mM EDTA, pH 8, 1.1 mM $MgCl_2$, 164 mM NaCl, 9 mM KCl, 0.82% SDS, 0.2 mg/ml yeast tRNA, 0.9% glycerol and 0.14 mg/ml proteinase K. The mixture was extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, dissolved in water and reprecipitated in the presence of $^{32}$P-labeled primer extending from +35 to +16 of the *hsp70* gene. This primer detects both paused and read-through transcripts (6). The primer was annealed to the RNA and extended by RNase H-free MMLV reverse transcriptase (Gibco BRL) as described by Li *et al*. (6). After ethanol precipitation the cDNA was dissolved in gel loading buffer and analyzed on a 7 M urea, 8% polyacrylamide gel. Bands representing the two major reverse transcripts were quantitated using a PhosphorImager® (Molecular Dynamics) and NIH Image software.

## RESULTS

As we had made no attempt to reconstitute nucleosomes in our crude extract, we questioned what role a nucleosome might have in pausing polymerase on the *hsp70* promoter in *Drosophila*. *A priori* the crude nature of our extract could not allow us to rule out the possibility that some nucleosomes might be assembling on the DNA template. To investigate whether a nucleosome is involved in pausing, we took advantage of two well-established facts. First, assembly of a nucleosome requires the wrapping of 146 bp around a histone core (11). Second, assembly of a nucleosome over the TATA box and the initiator inhibits transcription (12–14). We reasoned that if the DNA template was cut less than 146 bp from the transcription start and transcription still occurred on this linearized template a nucleosome could not be assembled on the region downstream of the transcription start. If downstream nucleosomes are indeed necessary for pausing, pausing should not occur on the digested DNA template.

Figure 1B provides evidence that polymerase still pauses even after the DNA has been cut at +103. Either closed circular or cut DNA (see Fig. 1A) was incubated in *Drosophila* nuclear extract under transcription conditions. The presence of paused polymerase was determined by monitoring the pattern of permanganate reactivity. Permanganate hyper-reactivity was evident at +22 and +30 for both the circular template (lanes 3 and 4) and the linear template (lanes 8 and 9). This hyper-reactivity was not evident when nucleotides were omitted (lanes 1, 5 and 6) or when α-amanitin was present (lanes 2 and 7). α-Amanitin inhibits elongation and prevents polymerase from elongating to the promoter-proximal region.

We extended our observation by determining if pausing still occurs when the DNA is cut 61 nt downstream of the transcription start (Fig. 1A). It is unlikely that any type of histone multimer could exist downstream of a paused polymerase on this template. Again, permanganate hyper-reactivity is evident at +22 for both the circular and linear templates (Fig. 1C, compare lanes 2 and 8 with lanes 1 and 7 respectively).

To demonstrate that the signals at +22 and +30 are dependent on the amount of DNA modified by $KMnO_4$, 80 and 20% of the original $KMnO_4$-treated transcription reactions were analyzed by primer extension. As expected, a significant difference in signal intensity was observed between the two amounts (Fig. 1C, compare lanes 2 and 7 with lanes 3 and 8).

So far, our results are consistent with the hypothesis that a nucleosome is not required to stably pause polymerase on the *hsp70* promoter. However, similar results might also be obtained if polymerase were to pause transiently and reinitiation were to occur at a sufficient rate to fill the paused position whenever a paused polymerase vacated this position.

To address whether the polymerases that we detect are transiently or stably paused, we determined if reinitiation occurs on our template in our reactions. Our previous analysis had indicated that reinitiation was not occurring on uncut templates under our reaction conditions (6). The experiment diagrammed in Figure 2A was done to determine if the cut templates also lacked reinitiation. A preinitiation complex was formed on the promoter for 25 min before addition of NTPs. At 6 or 30 min after addition of NTPs, the level of initiated transcripts was assayed by reverse transcription using a primer spanning the region from +35 to +16 (Fig. 2B) and quantitated (Fig. 2C). For both *Hin*dIII-digested and undigested constructs, the level of transcripts at 30 min was

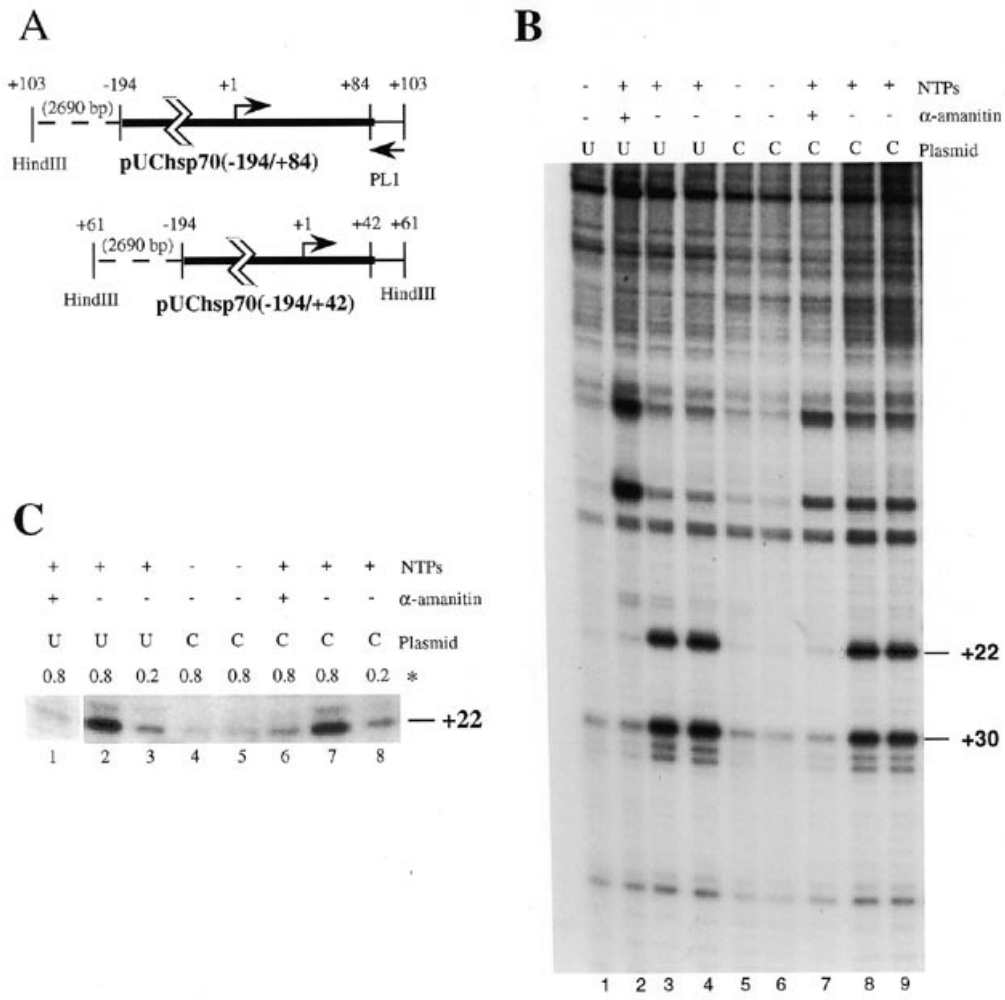


**Figure 1.** Effect of cutting the template on pausing on *hsp70 in vitro*. (**A**) Map of plasmids pUC*hsp70*(–194/+89) and pUC*hsp70*(–194/+42) with position of primer PL1. Cutting at the *Hin*dII site leaves a 19 nt extension beyond the end of the *hsp70* sequence. (**B**) pUC.XBS.*hsp70*(–194/+84) was cut with *Hin*dIII and protected from nucleases by dATPαS incorporation as described in Materials and Methods (lanes 5–9). Uncut plasmid was used as a control (lanes 1–4). *In vitro* transcription reactions were carried out in the presence or absence of 0.3 mM dNTPs. α-Amanitin (5 µg/ml) was included in the indicated reactions to inhibit Pol II transcription. The samples were treated with 33 mM $KMnO_4$ and the templates assayed for $KMnO_4$ modification by extension of $^{32}$P-labeled primer PL1. (**C**) pUC.XBS.*hsp70*(–194/+42) was digested with *Hin*dIII and protected from nucleases by dATPαS incorporation (lanes 4–8). Uncut plasmid was used as a control (lanes 1–3). *In vitro* transcription reactions were carried out in the presence or absence of 0.3 mM dNTPs and the samples were treated with 33 mM $KMnO_4$. The templates were assayed for $KMnO_4$ modification by extension of $^{32}$P-labeled primer PL-1 after they were diluted to the indicated (*****) proportion of their original concentration to confirm the dependence of signal intensity on template concentration.

equivalent to the level of transcripts at 6 min (Fig. 2C). To determine whether this was due to a lack of reinitiation or due to degradation of RNA produced early in the time course, α-amanitin was added 3 min after NTP addition to stop RNA production. The level of RNA at 30 min after addition of NTPs was found to be equal to that at 6 min after addition of NTPs, demonstrating that no significant loss of RNA occurs during the time course of the experiment.

The level of transcript produced by the linearized template was consistently less than the level from the uncut template. The result in lane 14 indicates that this decrease in signal is likely to be due to inactivation of the linearized template. Preincubating the template for 49 min prior to adding NTPs results in a level of transcript that is ~25% of the amount detected from uncut template. This result and the measurement from the 25 min preincubation are consistent with the linearized template having a half-life of ~25 min. Inactivation of the linearized template is most likely due to exonuclease activity (15). Importantly, the rate of template inactivation is not sufficient to negate our conclusion that there is no reinitiation in our reactions. Because reinitiation does not occur, the polymerase observed in the region that includes +22 and +30 can only be the result of a single initiation and stable pausing event.

## DISCUSSION

We conclude from our experiments that nucleosomes are not required for pausing RNA polymerase II on the *hsp70* promoter of *Drosophila*. This conclusion is consistent with recent high resolution analyses of the DNase I cutting pattern observed on this

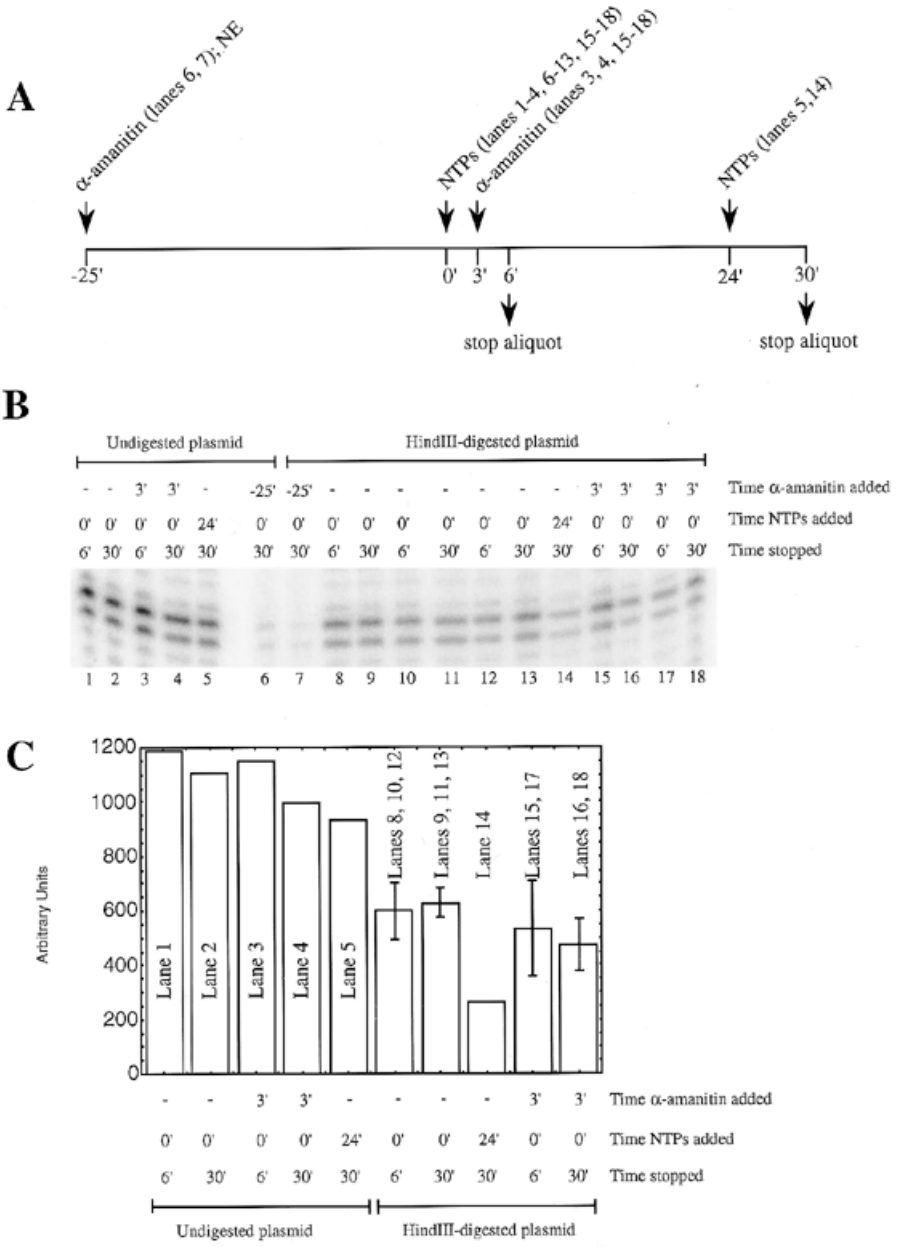


**Figure 2.** Kinetic analysis of the reinitiation of polymerase on *Hin*dIII-digested pUC.XBS.*hsp70*(–194/+42). (**A**) Schematic of kinetic analysis. *Drosophila* nuclear extract, salts and undigested (lanes 1–6) or *Hin*dIII-digested (lanes 7–18) pUC.XBS.*hsp70*(–194/+42) were added to all reactions at –25 min. NTPs and α-amanitin were added to the reactions and aliquots of the reactions were added to transcription stop buffer at the times indicated. Transcripts were visualized on a 7 M urea/8% polyacrylamide gel (**B**) after reverse trancription using $^{32}$P-labeled primer extending from +35 to +16. (**C**) Quantitative analysis of reverse transcripts. The two major reverse transcription products in (B) were quantitated as described in Materials and Methods. The values obtained for lanes 6 and 7 were used to correct the data from lanes 1–5 and 8–18 respectively for reverse transcripts resulting from *hsp70* RNA already present in the nuclear extract. Where applicable the graph shows the mean and standard deviation of identical reactions.

promoter in isolated nuclei (7,16). Strong cutting is observed in the region encompassing +65, suggesting that this region is nucleosome free.

Results from analysis of the c-*myc* promoter are also consistent with the notion that pausing does not require a nucleosome. In this case paused polymerase can be released in nuclei by treatment with 150 mM KCl (1). In contrast, a KCl concentration in excess of 250 mM was required in a reconstituted system for polymerase to resume elongation when it had first been paused by a nucleosome (17). That a nucleosome is not involved in pausing polymerase in the promoter-proximal region of c-*myc* is also consistent with the finding that the first 100 nt downstream of the transcription start are readily cut by micrococcal nuclease in isolated nuclei (18).

How can our results and those from past studies be reconciled with the evidence that a nucleosome is involved in pausing polymerase on the *hsp70* promoter in human cells? One possibility is that while nucleosomes are not necessary for the

pausing of polymerase to be stable in the time frame (30 min) we studied, they may help stabilize pausing beyond that point or may regulate the release of paused polymerase in response to certain activators. Another possibility is that there are different mechanisms for pausing polymerase, with nucleosomes playing a role in pausing on some promoters. In the case of the c-*fos* promoter permanganate footprinting showed that there are two regions of paused polymerase (3). One is situated near the promoter in a region 30–47 nt downstream of the start. The other appears to be located 385 nt downstream of the transcription start in a region encompassing a T-rich stretch. It remains to be determined whether the mechanism for pausing in these two locations differs, but certainly this is a strong possibility.

It is also possible that the pausing generated by Brown and colleagues is not a true reflection of what is happening in the cell. The generation of a paused polymerase on the human *hsp70* promoter by Brown and colleagues was almost unavoidable given the experimental manipulations that they employed (5). An elongation complex pausing at +15 was first generated by performing the transcription reaction in the absence of one nucleotide. Then the DNA template was stripped of most factors by 1% sarkosyl, leaving an essentially naked DNA template with a paused polymerase and a nascent transcript. Nucleosomes were then assembled on this template in a *Xenopus* extract. It is not surprising that nucleosomes assemble on the region flanking the RNA polymerases. Based on previous work by Isban and Luse (17) one would fully expect that these nucleosomes will cause polymerase to pause, whether they are of physiological relevance or not. While one *in vitro* pausing site detected by Brown *et al*. (5) corresponded to the location where permanganate footprinting indicated that polymerase was paused *in vivo*, other sites of pausing at +20 in their reconstituted reactions did not. This dicotomy again points to the possibility that different mechanisms could be the basis for pausing on various promoters.

It is important to note that the chromatin-dependent pause reconstituted on the human *hsp70* promoter correlates with a transient pause that is observed on a naked DNA template. Previous studies clearly indicate that the rate of elongation by RNA polymerase II is influenced by the underlying sequence (19,20). The duration of pauses could be lengthened by a variety of factors, including nucleosomes, sequence-specific DNA binding proteins and other DNA binding molecules (5,17,21,22). Indeed, it is possible that individual histones, possibly present in our extracts, could still bind DNA and provide some resistance to polymerase movement. The results presented here indicate that a downstream nucleosome is not a universal requirement for stably pausing RNA polymerase in the promoter-proximal region.

## ACKNOWLEDGEMENT

This work was supported by National Insititutes of Health grant GM47477.

## REFERENCES

1 Krumm,A., Meulia,T., Brunvand,M. and Groudine,M. (1992) *Genes Dev.*, **6**, 2201–2213.
2 Mirkovitch,J. and Darnell,J.E.J. (1992) *Mol. Biol. Cell*, **3**, 1085–1094.
3 Plet,A., Eick,D. and Blanchard,J.M. (1995) *Oncogene*, **10**, 319–328.
4 Rasmussen,E.B. and Lis,J.T. (1995) *J. Mol. Biol.*, **252**, 522–535.
5 Brown,S.A., Imbalzano,A.N. and Kingston,R.E. (1996) *Genes Dev.*, **10**, 1479–1490.
6 Li,B., Weber,J.A., Yan,C., Greenleaf,A.L. and Gilmour,D.S. (1996) *Mol. Cell. Biol.*, **16**, 5433–5443.
7 Weber,J.A., Taxman,D.J., Lu,Q. and Gilmour,D.S. (1997) *Mol. Cell. Biol.*, **17**, 5433–5443.
8 Biggin,M.D. and Tjian,R. (1988) *Cell*, **53**, 699–711.
9 Emanuel, P.A. and Gilmour, D.S. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 8449–8453.
10 Sandaltzopoulos,R., Blank,T. and Becker,P.B. (1994) *EMBO J.*, **13**, 373–379.
11 Ramakrishnan,V. (1997) *Annu. Rev. Biophys. Biomol. Struct.*, **26**, 83–112.
12 Lorch,Y., W,L.J. and Kornberg,R.D. (1987) *Cell*, **49**, 203–210.
13 Workman,J.L. and Roeder,R.G. (1987) *Cell*, **51**, 613–622.
14 Becker,P.B., Rabindran,S.K. and Wu,C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4109–4113.
15 Gilmour,D.S., Dietz,T.J. and Elgin,S.C.R. (1988) *Mol. Cell. Biol.*, **8**, 3204–3214.
16 Weber,J.A. and Gilmour,D.S. (1995) *Nucleic Acids Res.*, **23**, 3327–3334.
17 Izban,M.G. and Luse,D.S. (1991) *Genes Dev.*, **5**, 683–696.
18 Wolf,D.A., Strobl,L.J., Pullner,A. and Eick,D. (1995) *Nucleic Acids Res.*, **23**, 3373–3379.
19 Kerppola,T.K. and Kane,C.M. (1990) *Biochemistry*, **29**, 269–278.
20 Kephart,D.D., Marshall,N.F. and Price,D.H. (1992) *Mol. Cell. Biol.*, **12**, 2067–2077.
21 Reines,D. and Mote,J.J. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 1917–1921.
22 Mote,J.J., Ghanouni,P. and Reines,D. (1994) *J. Mol. Biol.*, **236**, 725–737.