MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5433–5443 Vol. 16, No. 10
0270-7306/96/$04.00+0

Analyses of Promoter-Proximal Pausing by RNA Polymerase II on the *hsp70* Heat Shock Gene Promoter in a *Drosophila* Nuclear Extract

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Received 13 February 1996/Returned for modification 5 May 1996/Accepted 2 July 1996

Analyses of *Drosophila* cells have revealed that RNA polymerase II is paused in a region 20 to 40 nucleotides downstream from the transcription start site of the *hsp70* heat shock gene when the gene is not transcriptionally active. We have developed a cell-free system that reconstitutes this promoter-proximal pausing. The paused polymerase has been detected by monitoring the hyperreactivity of thymines in the transcription bubble toward potassium permanganate. The pattern of permanganate reactivity for the *hsp70* promoter in the reconstituted system matches the pattern found on the promoter after it has been introduced back into flies by P-element-mediated transposition. Matching patterns of permanganate reactivity are also observed for a non-heat shock promoter, the histone H3 promoter. Further analysis of the *hsp70* promoter in the reconstituted system reveals that pausing does not depend on sequence-specific interactions located immediately downstream from the pause site. Sequences upstream from the TATA box influence the recruitment of polymerase rather than the efficiency of pausing. Kinetic analysis indicates that the polymerase rapidly enters the paused state and remains stably in this state for at least 25 min. Further analysis shows that the paused polymerase will initially resume elongation when Sarkosyl is added but loses this capacity within minutes of pausing. Using an alpha-amanitin-resistant polymerase, we provide evidence that promoter-proximal pausing does not require the carboxy-terminal domain of the polymerase.

RNA polymerase progresses through a sequential series of steps in the process of transcribing a gene. These steps include the recruitment of polymerase to the promoter, initiation, promoter clearance, elongation, and termination. Transcriptional regulation could occur at any of these steps. In bacteria, regulatory mechanisms that act subsequent to initiation are quite common (3). For protein-encoding genes in eukaryotes, the results of biochemical analyses have focused on steps associated with the recruitment and initiation of RNA polymerase II. For example, the model activator Gal4-VP16 has been found to increase promoter associations of TFIID (39) and TFIIB (4). However, the analysis of the density of RNA polymerase II associated with several different genes in cells indicates that the regulatory step for some genes may occur after initiation (1). From nuclear run-on assays, the density of RNA polymerase found proximal to the promoter exceeds the density found in the body of a variety of *Drosophila* and mammalian genes (14, 15, 30). This result suggests that there is at least one rate-limiting step that follows transcriptional initiation.

One of the most extensively analyzed cases of promoter-proximal pausing is the *hsp70* promoter of *Drosophila melanogaster* (19). This gene is not transcribed under normal growth conditions. UV cross-linking, transcription run-on, and potassium permanganate footprinting reveal that a polymerase molecule has initiated transcription but paused in the region 20 to 40 nucleotides downstream from the transcription start site (reference 28 and references therein). The level of paused polymerase seems to depend on a GAGA element located upstream from the TATA box and on sequences located between −12 and +33 (16). The GAGA element binds a ubiquitous factor called the GAGA factor (2, 10). This factor appears to increase the accessibility of sequences in chromatin and therefore could play a role in rendering the promoter accessible to the transcription apparatus (34, 37). Sequences between −12 and +33 have been shown to be recognized by TFIID (6, 26, 36). The organization of the paused polymerase on the heat shock promoter probably allows the heat shock gene to be rapidly induced by stress such as heat shock. Release of paused polymerase is presumably coupled to transcriptional activation of the gene by the heat shock factor.

The basis for promoter-proximal pausing is not known. Recent analysis of the polymerase associated with the uninduced and heat shock-induced *hsp70* gene indicates that pausing could be related to the unusual carboxy-terminal domain (CTD) of the largest subunit in RNA polymerase II. The CTD is composed of a seven-amino-acid sequence that is repeated 42 times in a tandem array in *D. melanogaster*. The seven-amino-acid consensus sequence, Tyr-Ser-Pro-Thr-Ser-Pro-Ser, can be highly phosphorylated. In vivo cross-linking of *Drosophila* tissue culture cells and indirect immunofluorescence analysis of polytene chromosomes both indicate that the paused polymerase is primarily in the dephosphorylated state, whereas the polymerase associated with the body of the heat shock genes after induction is highly phosphorylated (22, 41). The dephosphorylated form of the CTD is found to have higher affinity for TATA-binding protein (TBP) than the phosphorylated form (35). These coincidences have lead to a model whereby the dephosphorylated form of the CTD might serve to tether the paused polymerase to TBP (14, 19). Release might be achieved by phosphorylating the CTD.

A significant limitation in studying promoter-proximal pausing in eukaryotes has been the lack of a cell-free system that reconstitutes pausing. We report the successful reconstitution

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of pausing on the *Drosophila hsp70* promoter in nuclear extracts from *Drosophila* embryos. We have used this system to investigate certain features of promoter-proximal pausing that have emerged from previous in vivo studies.

MATERIALS AND METHODS

In vitro transcription. *Drosophila* embryo nuclear extracts were made as described by Biggin and Tjian (2). In vitro transcription was carried out in 50 mM KCl–50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (K^+; pH 7.6)–6.25 mM $MgCl_2$–5% glycerol–0.5 mM dithiothreitol (DTT). The 40-μl reaction mixture contains 20 μl of extract, 10 ng of *hsp70* DNA template, 1 μg of *Hae*III-cut *Escherichia coli* DNA, and 0.3 mM nucleoside triphosphates (NTPs). The reaction mixture was incubated at 21°C for 30 min, and transcription was stopped by the addition of 80 μl of stop buffer (20 mM EDTA [pH 8], 0.2 M NaCl, 1% sodium dodecyl sulfate [SDS], 0.25 mg of yeast tRNA per ml, 0.1 mg of proteinase K per ml). After phenol extraction and ethanol precipitation, the nucleic acid was mixed with 0.03 pmol of radioactive primer in 10 μl of 2 mM Tris-Cl (pH 7.8)–0.2 mM EDTA–250 mM KCl. The mixture was heated to 75°C and allowed to cool to room temperature. Forty microliters of reverse transcription mixture containing 62.5 mM Tris-Cl (pH 8.3), 30 mM KCl, 3.75 mM $MgCl_2$, 12.5 mM DTT, 62.5 μM dNTPs, and 15 U of Moloney murine leukemia virus reverse transcriptase, RNase H^- (Promega), was added, and the mixture was incubated for 1 h at 37°C. The nucleic acid was ethanol precipitated and run on an 8% denaturing polyacrylamide gel.

Potassium permanganate analysis of pausing reconstituted in extracts. Transcription reactions were performed as described above. When used, alpha-amanitin was added to a final concentration of 4 or 5 μg/ml. After incubation for 30 min at 21°C, permanganate modification was performed by adding 5 μl of freshly made 300 mM $KMnO_4$. After 4 min (in Fig. 6, the reaction time was shortened to 2 min), the reaction was terminated by the addition of 200 μl of stop buffer containing 50 mM EDTA, 1% SDS, and 0.4 M β-mercaptoethanol. After phenol extraction and ethanol precipitation, the nucleic acid was added to a 100-μl PCR mixture containing 67 mM Tris-Cl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 100 μg of bovine serum albumin per ml, 200 μM dNTPs, 0.1 pmol radioactive primer, and 1.5 U of *Taq* polymerase. For the nontranscribed strand, a reverse sequencing primer was used. For the transcribed strand, a primer spanning positions −57 to −34 was used. Fifteen cycles of linear PCR were performed, and then nucleic acid was isolated by phenol extraction and ethanol precipitation. The nucleic acid was analyzed on 8% denaturing polyacrylamide gels. Note that the analysis of permanganate reactivity in Fig. 3B was done by using ligation-mediated PCR rather than the method just described. For ligation-mediated PCR, the DNA was processed as described below for genomic DNA.

Pausing with alpha-amanitin-resistant polymerase. The alpha-amanitin-resistant polymerase, C4, was prepared as described previously (45). Chymotrypsin treatment of the C4 polymerase was performed as follows. A 50-μl reaction was set up by mixing 10 μl of C4 polymerase (190 U/μl, 200 ng/μl), 25 μl of 2× reaction buffer (1× reaction mixture is 50 mM Tris [pH 7.4], 0.1 mM EDTA, 25% glycerol, and 0.5 mM DTT), 5 μl of chymotrypsin (25 μg/ml), and 10 μl of H_2O. The reaction was continued for 30 min at 25°C and was then stopped by adding 2 μl of a mix of protease inhibitors (500 μg of soybean trypsin inhibitor per ml and 20 mM phenylmethylsulfonyl fluoride); 4 μl of reaction mixture was then checked by SDS-polyacrylamide gel electrophoresis (PAGE) (5% gel) and silver staining. The reaction mixture was also checked for polymerase II activity by nonspecific in vitro transcription assay. In the $KMnO_4$ assay, 5 μl of the chymotrypsin-treated C4 polymerase was used in each reaction. For the intact polymerase reaction, 1 μl of C4 polymerase was added to a chymotrypsin reaction mixture in which the chymotrypsin had been inactivated by pretreatment with soybean trypsin inhibitor. In all cases, the C4 polymerase was preincubated with the nuclear extract on ice for 15 min before initiation of transcription with the addition of DNA and NTPs.

Fly transformation and genomic footprinting with potassium permanganate. Transformed lines containing *hsp70* promoter constructs were generated by using P-element-mediated transformation (31) and a vector called Car20-lacZT.2. Details on this transformation vector are given in reference 40. The *hsp70* promoter constructs were linked to the *E. coli* β-galactosidase gene by a synthetic linker that provides a ribosome binding site and a translation start. The DNase I hypersensitivity and levels of transcription, both basal and induced, for each of the independent lines were determined. The two lines chosen for each promoter construct had identical patterns of hypersensitivity and comparable levels of expression. All lines were maintained on standard cornmeal-yeast food at 25°C. For embryo collection, separate stocks of flies were set up in containers with removable trays. All embryos represent 0- to 18-h overnight collections. They were dechorionated, flash frozen in liquid nitrogen, and stored at −80°C until processed.

Nuclei were isolated from dechorionated embryos by using a shortened version of a protocol previously described (40). Dechorionated embryos were homogenized in 1 M sucrose buffer (60 mM KCl, 15 mM NaCl, 100 mM EGTA, 0.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 15 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 1 M sucrose) and pelleted by centrifugation for 10 min in an HB-4 rotor at 6,500 rpm in the cold. The nucleus pellet was resuspended in fresh 1 M sucrose buffer and layered onto a cushion of 1.7 M sucrose buffer. The interface was mixed, and tubes were centrifuged for 25 min in an HB-4 rotor at 10,000 rpm in the cold. The supernatant was carefully removed, and the nucleus pellet was suspended in 1× DNase I buffer (60 mM KCl, 15 mM NaCl, 0.5 mM DTT, 15 mM Tris-HCl [pH 7.4], 0.25 M sucrose, 3 mM $MgCl_2$) and held on ice. Treatment of nuclei with 25 mM potassium permanganate was performed for 30 s at 0°C and was stopped by the addition of EDTA and β-mercaptoethanol to 25 mM and 0.2 M, respectively (8). Naked DNA standards were prepared by using DNA purified from an equal aliquot of untreated nuclei and treating it with 25 mM potassium permanganate for 30 s at 0°C. DNA purified either from treated nuclei or naked DNA was dissolved in 10% piperidine, heated at 90°C for 30 min, and then ethanol precipitated twice. All samples were resuspended in 1 mM EDTA–10 mM Tris-HCl (pH 7.5) so that the concentration was 200 ng/5 μl.

The reactivity of genomic DNA treated with potassium permanganate was visualized with a modified ligation-mediated PCR protocol which substitutes Vent polymerase (New England Biolabs) for Sequenase (U.S. Biochemical) and *Taq* polymerase (21). One hundred nanograms of genomic DNA was used in each reaction. An equal amount of genomic DNA, treated with dimethyl sulfate and piperidine to serve as G-specific markers, was processed in parallel with the potassium permanganate samples. The primers used to amplify and visualize the transformed promoters were the same as used in the earlier footprinting study (40), but annealing temperatures were adapted for the new buffer-polymerase combination: 50°C for the first genomic primer, 58°C for the second genomic primer, and 62°C for the third genomic primer. In addition, the labeling cycles were modified to contain 2 pmol of radiolabeled primer, compared with 0.5 pmol in the previous study. One-fourth of the labeled material for each sample was run on a standard sequencing gel (8% acrylamide, 8 M urea).

RESULTS

Reconstitution of promoter-proximal pausing in a *Drosophila* nuclear extract derived from embryos. We were initially alerted to the possibility that promoter-proximal pausing might be occurring in our in vitro transcription reactions when we noticed differences in the intensities of the signals produced by two primers that we used to detect transcription in vitro. These primers were used in primer extension assays. A primer spanning sequences from +35 to +16 (+35/+16 primer) consistently gave stronger signals that a primer spanning the region from +71 to +43. In Fig. 1A, quantification shows that the signal for the +35/+16 primer is 1.6 times stronger than the signal for the +71/+43 primer (compare lanes 5 and 6 with lanes 7 and 8). Although this difference might be due to pausing, we needed to consider the possibility that the primers had different specific activities or behaved differently in the primer extension assay. To monitor for variations that could be inherent to the primers rather than the level of transcript, the primers were used in an extension analysis of total RNA isolated from heat-shocked *Drosophila* embryos. Under these circumstances, the bulk of the *hsp70* RNA is produced by polymerases that have been released from the paused position. Quantification of the signals showed that the signal for the +35/+16 primer is 1.8 times weaker than the signal for the +71/+43 primer (compare lanes 1 to 3 and lanes 2 to 4). Therefore, with this batch of primers, we need to correct for the weakness of the +35/+16 primer. When this is done, the amount of polymerase that extends far enough to be detected with the +71/+43 primer is approximately threefold less than the amount of polymerase that extends far enough to be detected with the +35/+16 primer.

The results of the primer extension analysis indicated that more than half of the polymerases that initiated from the *hsp70* promoter in vitro failed to extend more than 40 nucleotides downstream from the transcription start. These polymerases could have paused in a promoter-proximal position, or they could have prematurely terminated transcription. To detect the paused polymerase directly, we probed the DNA with potassium permanganate. If the polymerases have paused, they should still be associated with the DNA and a single-stranded region should be present. The single-stranded region associ-

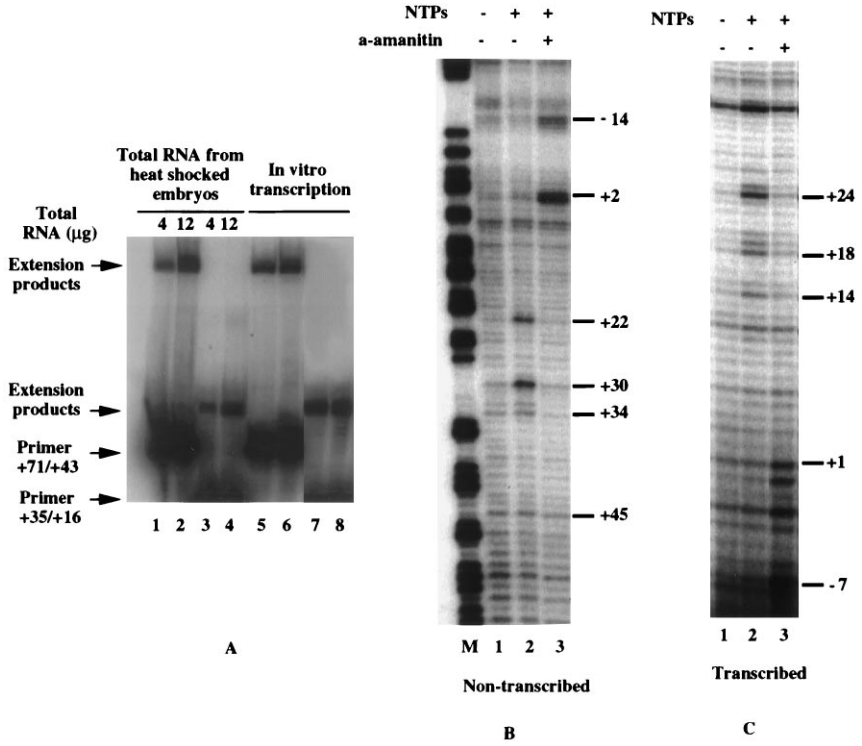

FIG. 1. Detection of paused polymerase on the *hsp70* promoter with permanganate. (A) Primer extension with two primers that anneal to *hsp70* RNA at different distances from the transcription start. RNA was detected by extending a primer that anneals to the region from +71 to +43 or from +35 to +16. Lanes 1 through 4 show primer extension of total RNA from heat-shocked embryos. Lanes 1 and 2 contain, respectively, 4 and 12 μg of RNA analyzed with the +71/+43 primer. Lanes 3 and 4 contain, respectively, 4 and 12 μg of RNA analyzed with the +35/+16 primer. Lanes 5 through 8 show primer extension of RNA produced in vitro by a *Drosophila* nuclear extract derived from non-heat-shocked embryos. Lanes 5 and 6 are duplicate samples that have been analyzed with the +71/+43 primer, and lanes 7 and 8 are duplicate samples that have been analyzed with the +35/+16 primer. (B) Permanganate reactivity on the nontranscribed strand. DNA and nuclear extract were incubated in the absence or presence of rNTPs (lanes 1 and 2, respectively). In lane 3, alpha-amanitin was added to 5 μg/ml at the beginning of the incubation. Paused polymerase was detected by reacting the mixture with permanganate at the end of 30 min. The pattern of reactivity was determined by using a primer extension analysis with *Taq* polymerase. Pausing is implicated by the hyperreactivity at +22 and +30 in lane 2. (C) Permanganate reactivity on the transcribed strand. The pattern of permanganate reactivity was detected by primer extension with a primer that annealed to the transcribed strand upstream from the transcription start.

ated with the polymerase that pauses on the *hsp70* promoter in *Drosophila* cells and in isolated nuclei can be detected with permanganate (8, 40). Thymines in single-stranded regions are hyperreactive to oxidation by permanganate, and these modified residues can be mapped with a primer extension protocol using *Taq* polymerase (38, 39). When we treated the in vitro transcription reaction with permanganate after incubating the extract with template and NTPs for 30 min, hyperreactive thymines were detected at +22 and +30 on the nontranscribed strand (Fig. 1B, lane 2). This pattern of hyperreactivity closely matches the pattern previously detected in intact *Drosophila* tissue culture cells (7, 8) and in nuclei isolated from *Drosophila* embryos (40). The hyperreactive sites were not detected if NTPs were omitted from the transcription reaction (Fig. 1B, lane 1). All four NTPs would be required for the polymerase to elongate into the paused position. The hyperreactivity at +22 and +30 was also absent when alpha-amanitin was included at 5 μg/ml in the reaction (Fig. 1B, lane 3). At this low concentration, alpha-amanitin specifically inhibits elongation of RNA polymerase II. Instead, the presence of alpha-amanitin caused the permanganate reactivity to occur at nucleotides −14 and +2 in the promoter. This is due to RNA polymerase molecules that have unwound the transcription start but are unable to elongate.

The pattern of permanganate sensitivity was also evaluated for the transcribed strand by using a primer that annealed upstream from the transcription start site. Hyperreactivity was detected around +14, +18, and +24 (Fig. 1C, lane 2). This pattern coincides exactly with the pattern reported for this strand of the *hsp70* promoter in *Drosophila* tissue culture cells (8). This downstream pattern of hyperreactivity was not evident when NTPs were omitted from the reaction or when alpha-amanitin was included in the reaction (Fig. 1C, lanes 1 and 3). With alpha-amanitin present, hyperreactivity was observed near the transcription start and upstream at −7; this pattern is consistent with polymerase unwinding the transcription start but failing to elongate.

To further verify that the permanganate hyperreactivity was due to the paused polymerase, we analyzed a series of reactions in which the polymerase would be artificially paused by the absence of one or more nucleotides. ATP alone allows the polymerase to unwind DNA. ATP and UTP allow the polymerase to advance to +3, whereas ATP, UTP, and CTP allow the polymerase to advance to +15. Comparison of the permanganate patterns shows that the peak of permanganate reactivity advances downstream in accord with the expected progress of the polymerase (Fig. 2A and B, lanes 2 to 4). When all four nucleotides are present, however, a significant level of permanganate reactivity remains in positions +22 and +30 on the nontranscribed strand and +24 on the transcribed strand (Fig. 2A and B, lanes 5). Hence, although the nucleotides are available for the polymerase to elongate farther downstream, a significant portion pauses in a promoter-proximal position. Importantly, the permanganate hyperreactivity indicates that

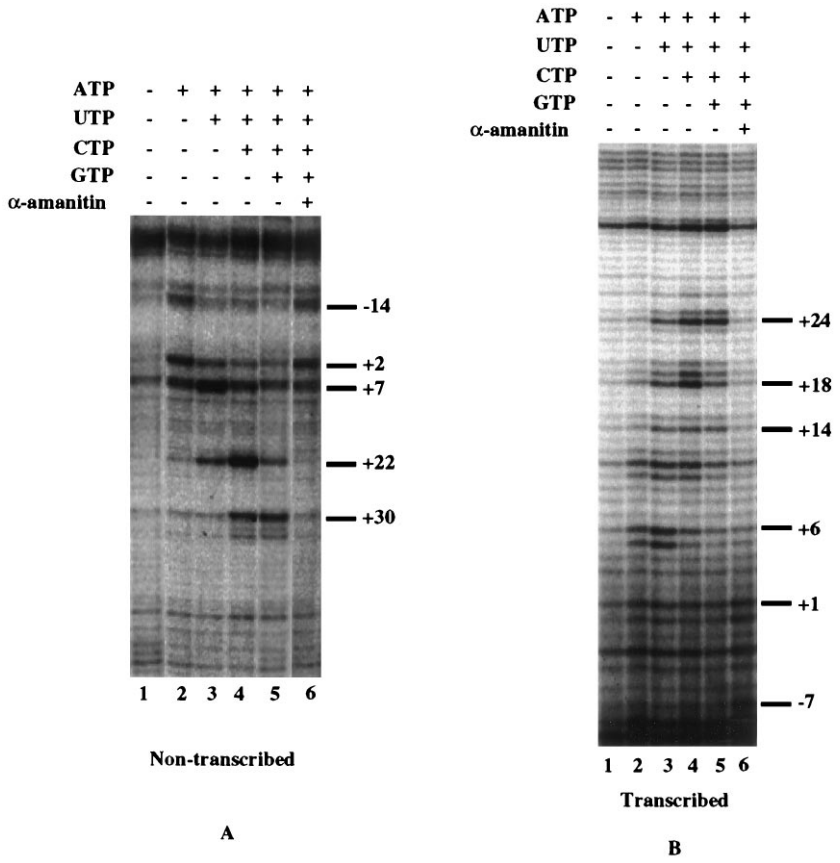

FIG. 2. Permanganate analysis of polymerase molecules that have paused at different distances from the start site. (A) Permanganate hypersensitivity on the nontranscribed strand. Different combinations of NTPs were added at the beginning of the incubation (lanes 2 to 4). ATP alone allows for unwinding at the transcription start. ATP and UTP allow for elongation to +3. ATP, UTP and CTP allow for elongation to +15. Paused polymerase was observed when all four nucleotides were present (lane 5). In lane 6, alpha-amanitin was added at the beginning of the incubation. (B) Permanganate hypersensitivity on the transcribed strand. The manipulations were the same as for panel A except that a different primer was used in the extension reactions with *Taq* polymerase.

these polymerases are engaged on the template rather than prematurely terminating and dissociating from the template.

Sequence dependence of promoter-proximal pausing. We were interested in defining the region of the promoter that was required for pausing in vitro and comparing this with the behavior of the promoters in vivo. A promoter can be assessed in vivo by transforming the promoter constructs into *Drosophila* cells by using P-element-mediated transformation and then analyzing the pattern of permanganate reactivity that is apparent in nuclei isolated from the transformed embryos (40). Constructs containing the promoter region from −194 to +84 and −89 to +84 produced robust permanganate reactivity at +22 and +30 both in the reconstituted reaction and in nuclei from embryos (Fig. 3A, lanes 1 and 3; Fig. 3B, lanes 4 and 6). In contrast, the construct containing the promoter region from −50 to +84 exhibited substantially less permanganate reactivity for both the in vitro and in vivo situations. This series of deletions progressively removes more and more GAGA elements (10). It has been shown previously that a mutation in a GAGA element located at −70 causes a reduction in the level of paused polymerase found on an *hsp70* promoter construct that extends from −89 to +62 (16).

The reduction in paused polymerase caused by the deletion of sequences between −89 and −50 could be due to decreases in the recruitment of polymerase or the efficiency of pausing. To determine which of these occurred in vitro, the effects of alpha-amanitin were examined. We reasoned that the level of permanganate reactivity at the transcription start would provide a relative measure of the level of polymerase recruited to different promoters. Figure 3A shows that deletion of sequences between −89 and −50 results in approximately a fourfold reduction in the level of permanganate reactivity observed at +2 (compare lanes 4 and 6). This is comparable to the decrease observed at +22 when alpha-amanitin is omitted (compare lanes 3 and 5). Together, these results suggest that the sequences upstream from −50 contribute primarily to the recruitment of polymerase rather than the efficiency of pausing.

It should be noted that the pattern of permanganate reactivity in Fig. 3B was monitored differently than in Fig. 3A. To detect the reactivity on a single-copy gene in vivo, the permanganate reacted sites in the DNA were cleaved with piperidine. This was followed by ligation-mediated PCR. Under these conditions, +22 and +30 remain the most reactive sites (Fig. 3B). In addition, reactivity at +34 and +45, which was not readily apparent in the previous experiments, was also very evident. This discrepancy appears to be due to differences in the methods of visualizing the permanganate reactivity. When permanganate-reacted DNA from the reconstituted reaction was analyzed by using piperidine cleavage and ligation-mediated PCR, reactivity at +34 and +45 was readily apparent (Fig. 3B, lane 8).

We also analyzed the contribution of sequences downstream from the transcription start site. Lee et al. have shown that

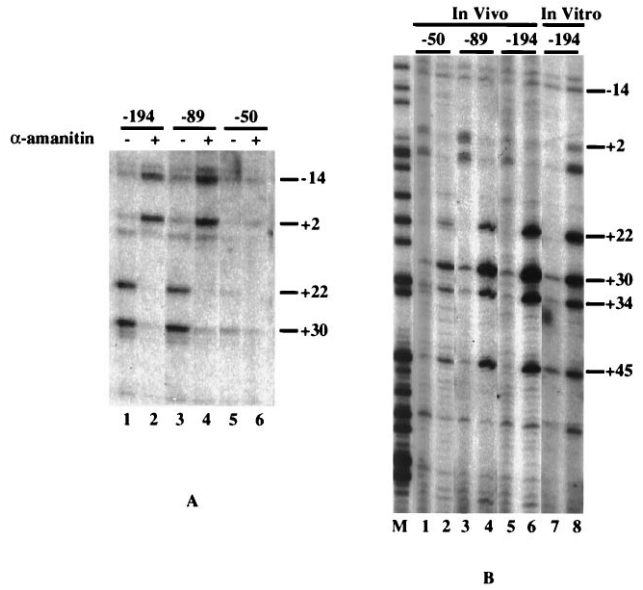

FIG. 3. Effects of upstream sequences on pausing in vitro and in vivo. (A) Permanganate analyses were performed on three different constructs of the *hsp70* promoter that were incubated in extract in the presence and absence of alpha-amanitin. The constructs extended to −194, −89, and −50 before connecting to vector sequences. All three promoters extend to +84 before linking to the coding region of β-galactosidase. Permanganate modifications on the nontranscribed strand were detected by using a primer that annealed downstream from the transcription start. (B) Permanganate reactivity of promoter constructs in nuclei. The three promoter constructs corresponding to those analyzed in vitro were transformed into flies on P elements. Nuclei were isolated from embryos of transformed flies and treated with permanganate (lanes 2, 4, and 6). Permanganate reactivity of purified genomic DNA is shown in lanes 1, 3, and 5. The pattern of modification was determined by ligation-mediated PCR as described in Materials and Methods. For comparison, ligation-mediated PCR was also used to detect the pattern of permanganate reactivity associated with DNA that had been incubated in extract in the absence (lane 7) and presence (lane 8) of NTPs.

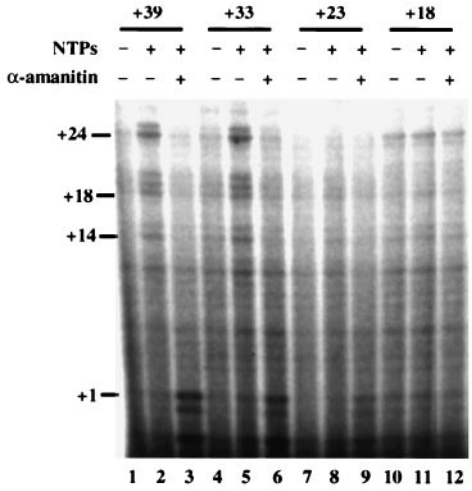

FIG. 4. Effects of 3′ deletions on pausing. Permanganate analysis was done on a series of *hsp70* constructs with the 3′ deletion breakpoints at +39, +33, +23, and +18 and a common 5′ end at −194. Permanganate modifications on the transcribed strand were detected by using a primer extension with *Taq* polymerase. The primer annealed to the transcribed strand upstream from the TATA box.

changes in the sequences downstream from +33 in the *hsp70* promoter decrease the level of paused polymerase by only 30%, whereas the deletion of sequences between +33 and −12 eliminates detection of paused polymerase (16). Members of a 3′ deletion series were incubated in the extract and then analyzed for pausing with potassium permanganate. The transcribed strand was analyzed so that the modifications that were detected by primer extension aligned on the sequencing gel. Constructs with breakpoints at +39 and +33 exhibited comparable patterns and levels of permanganate reactivity on the transcribed strand (Fig. 4, lanes 2 and 5). These results indicate that the sequence composition immediately adjacent to where pausing occurs has no detectable impact on the efficiency of pausing in vitro.

Deletions with breakpoints at +23 and +18 were also analyzed. These deletions exhibited noticeably less reactivity than the +39 and +33 deletions (Fig. 4, lanes 8 and 11). The most striking change, loss of the reactivity at +24, can be accounted for by the change in sequence. There was, however, still apparent a low level of permanganate reactivity at +18, +19, and +20 on the +23 deletion construct, indicating that pausing occurred. The reactivity at these positions diminished when NTPs were omitted or when alpha-amanitin was present (Fig. 4; compare lane 8 with lanes 7 and 9). Comparison of the reactivity found at +1 when alpha-amanitin is present (lanes 3, 6, and 9) suggests that the deletion of sequences between +33 and +23 inhibits primarily the recruitment of polymerase rather than the efficiency of pausing. The +18 deletion had debilitated the promoter enough so that the interaction of polymerase could not be detected under any conditions with permanganate, in accord with the reduced affinity that this promoter has for TFIID (6).

Pausing on the histone H3 promoter in vitro and in vivo. We recently reported that paused polymerase could not be detected on the endogenous histone H3 promoter in nuclei from *Drosophila* embryos (40). Therefore, if the reconstituted system reflected the pausing mechanism that occurred in vivo, we predicted that paused polymerase would not be apparent on the histone H3 promoter in vitro. We found that contrary to this prediction, paused polymerase was apparent; permanganate reactivity is detected at thymines located at +10, +17, +22, +25, +31, and +38 (Fig. 5, lane 2). The endogenous copies of the histone H3 promoter are present in approximately 100 copies in the fly genome (18). The discrepancy between the behavior of the cloned promoter in vitro and the natural promoters in vivo might be due to heterogeneity in the activity of different copies. Indeed, UV cross-linking analysis has provided evidence that a significant proportion of the histone genes can be transcriptionally inactive in tissue culture cells (9). Because of the uncertainties associated with the endogenous histone promoters, we analyzed the permanganate pattern of a transformed version of the histone H3 promoter, which could be readily distinguished from the endogenous copies because the promoter had been fused to the β-galactosidase gene. Permanganate analysis of this transformed promoter revealed hyperreactive sites that match well with the pattern observed for this promoter in vitro (Fig. 5, lane 4). Hence, two promoters, the *hsp70* and histone H3 promoters, exhibit similar patterns of permanganate reactivity when compared in vitro and in vivo.

In vitro, the paused polymerase is a stable complex. We were interested in determining if the paused polymerase was a stable complex or an intermediate in a dynamic process. The dynamic process might involve a situation in which polymerase prematurely terminates or resumes elongation but is rapidly replaced by reinitiation. Dynamic cycling of the polymerase has been shown to occur in prokaryotes (17) and has been suggested for eukaryotes (12). To address this point, we per-

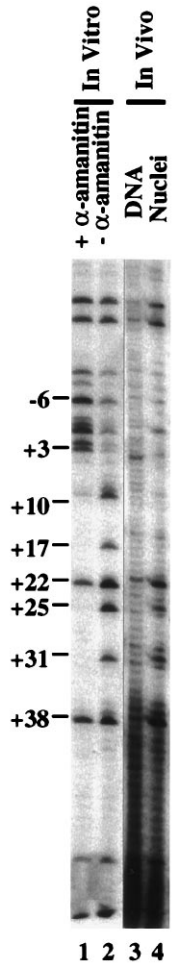

FIG. 5. Polymerase pausing on the H3 promoter in vitro and in vivo. The H3 promoter region spanning from −117 to +56 was subcloned into the Car20-lacZT.2 P-element transformation vector. Lanes 1 and 2 show the pattern of permanganate reactivity detected on the H3 promoter region when the plasmid was incubated with extract in the absence and presence of alpha-amanitin. Lanes 3 and 4 show the pattern of permanganate reactivity detected on the H3 promoter after it had been transformed into the genome by P-element-mediated transformation. Lane 3 is the pattern of reactivity for isolated genomic DNA, and lane 4 is the pattern of reactivity in nuclei.

formed the following kinetic analysis (Fig. 6A). First, preinitiation complexes were assembled on the template by incubating template and extract in the absence of NTPs. After 25 min, transcription was initiated by adding all four NTPs. Beginning 1 min after initiation of transcription, paused polymerase was detected by monitoring permanganate reactivity. Figure 6B shows the results of a representative experiment, and Fig. 6C and D compile the results of three independent experiments. The permanganate hyperreactivity at +22 and +30 is detected within 1 min of initiation of transcription, and it persists at this level for at least 25 min.

The kinetics of pausing suggested that the polymerase rapidly entered the paused position and then remained there for at least 25 min. Alternatively though, one could envision that a polymerase paused for a short period of time but was then rapidly replaced by a new polymerase when and if the preceding polymerase resumed elongation or prematurely terminated transcription. To distinguish between these possibilities, we analyzed the rate of transcript synthesis. Figure 6E shows a representative result, and Fig. 6F compiles the results of three independent experiments. The peak level of transcripts was reached in 1 min and remained unchanged thereafter (Fig. 6E, lanes 1 to 4). There did not appear to be any turnover of the message, since the level of transcript remained constant even when transcription was inhibited by adding alpha-amanitin 1 min after initiation of transcription (lanes 5 to 7). Hence, only one round of initiation occurred under these reaction conditions.

Paused polymerase rapidly loses its ability to resume elongation when released by Sarkosyl. Detailed analysis of the *hsp70* transcripts found in nuclei from non-heat-shocked cells identified two types of short transcripts (28). One appears to be associated with elongation-competent forms of paused polymerase. These increase in length when NTPs and Sarkosyl are added to the nuclei. The second type of transcript does not increase in length when NTPs and Sarkosyl are added. The latter transcripts could be associated with polymerase molecules that are incapable of resuming elongation when the NTPs and Sarkosyl are added, or they may represent transcripts that have been released from the DNA template. In nuclei, the former type of transcript appears to be more abundant than the latter type.

The observations of Rasmussen and Lis (28) prompted us to investigate how the polymerase that had paused in the crude extract would respond to the addition of Sarkosyl. At first, we focused on the behavior of the paused polymerase by monitoring permanganate reactivity. Paused polymerase was formed upon incubation of the promoter template with extract and NTPs for 29 min. When Sarkosyl was added to the paused polymerase, the permanganate reactivity at +22 and +30 diminished greatly (Fig. 7A, lane 4). This result indicates that the paused polymerase had been released from the DNA template by the detergent. The effect of alpha-amanitin was examined to determine if the release was through an elongation-dependent step. Alpha-amanitin was found to block the effect of Sarkosyl when the alpha-amanitin was added to the paused polymerase 1 min before the addition of Sarkosyl (Fig. 7A, lane 5). This result suggests that the Sarkosyl release involves an elongation-dependent step.

We next determined if the released polymerase was elongation competent. If it was, then the released polymerase should elongate the transcript to a length that could be detected with the +71/+43 primer. However, we consistently observed that addition of Sarkosyl failed to cause any detectable increase in the signal detected by the +71/+43 primer when transcripts were analyzed from samples similar to those resulting in lane 4 of Fig. 7A (data not shown). Hence, the polymerase that was released by Sarkosyl was unable to elongate any substantial distance.

The failure of the polymerase to resume elongation in our reactions was reminiscent of results previously reported by Marshall and Price (20). Their analysis of radiolabeled transcripts showed that the majority of the polymerase initiating on the actin promoter in a *Drosophila* extract paused in the promoter-proximal region. These polymerases would resume elongation if KCl was added within 1 min of initiation. This capacity to resume elongation was lost over a period of several minutes.

We performed the following kinetic analysis to determine if the polymerases that paused in our reaction changed from an elongationally competent form to an incompetent form. Preinitiation complexes were formed by incubating DNA with extract for 30 min. Transcription was started by the addition of NTPs. Sarkosyl was added at various times after initiation, and then the mixture was incubated for an additional 10 min to

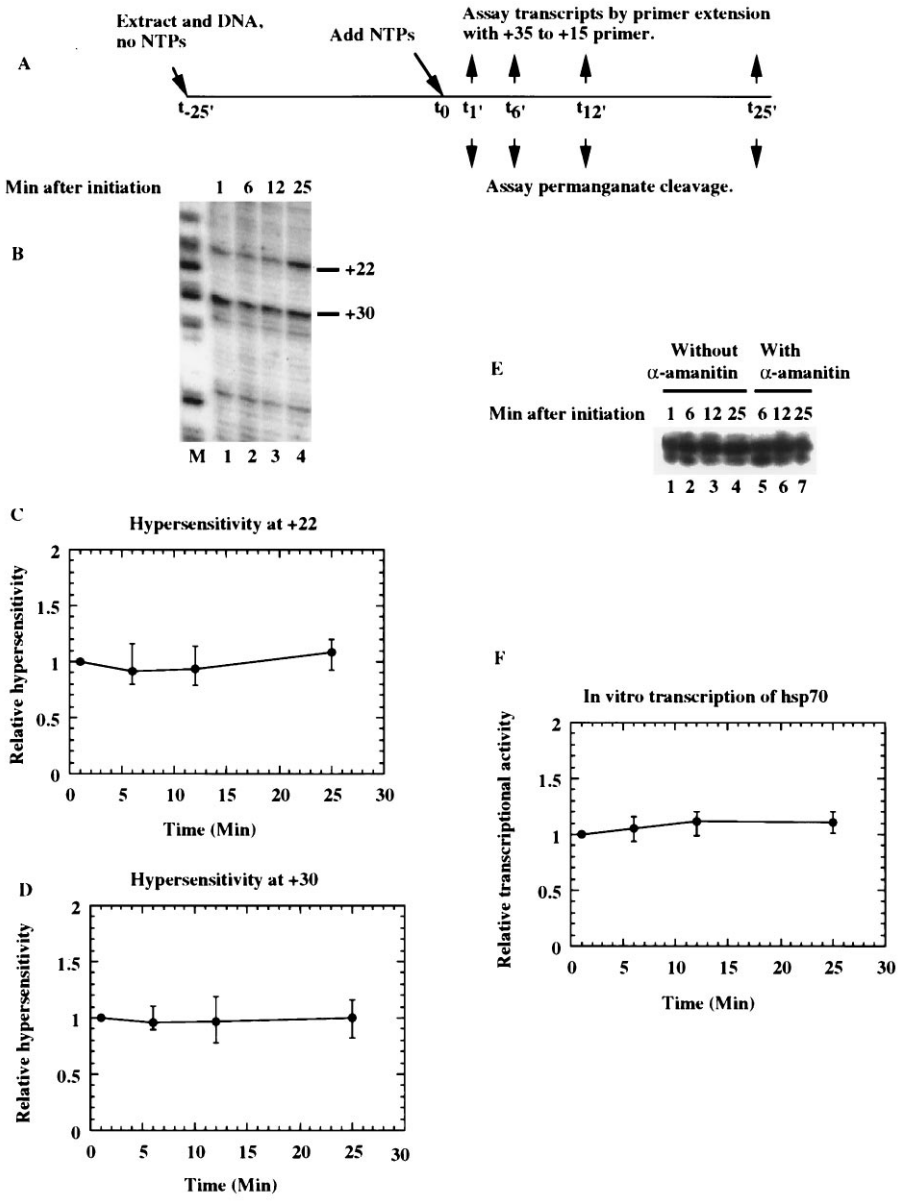

FIG. 6. Kinetic analyses of polymerase pausing on the *hsp70* promoter. (A) Experimental design. Preinitiation transcription complexes were assembled in the absence of rNTPs. After 25 min, rNTPs were added to a final concentration of 0.3 mM. At various times after the addition of rNTPs, aliquots of the reaction were taken to do either permanganate analysis or transcript measurements. (B) Permanganate analysis of the aliquots taken at time 1, 6, 12, and 25 min after nucleotide addition. (C) Quantitation of permanganate reactivity at +22 for three independent experiments. The permanganate-sensitive band at +22 was quantitated with a PhosphorImager. For a given experiment, the values were normalized to that for the 1-min time point and plotted against time. (D) Quantitation of permanganate reactivity at +30 for three independent experiments. Quantitative analysis was performed as described for panel C. (E) Transcript analysis. Aliquots of the transcription reaction used for panel A were stopped 1, 6, 12, and 25 min after the addition of nucleotides (lanes 1 to 4). Short transcripts were detected by using primer extension with reverse transcriptase and a primer that is complementary to the region from +35 to +15 in the transcript. To determine if there was any turnover of the RNA, a second time course was performed on samples that were inhibited by adding alpha-amanitin (5 μg/ml) 1 min after initiation of transcription (lanes 5 to 7). (F) Quantitation of transcript levels. Only the transcription levels without alpha-amanitin were quantitated with a PhosphorImager, and the values for each experiment were normalized to the level detected at 1 min. The graphs compile the data from three separate experiments.

allow time for elongation. A control sample for each time point lacked the Sarkosyl addition. Elongation by polymerase beyond the promoter-proximal position was monitored by primer extension with the +71/+43 primer. When Sarkosyl was added 1 min after the start of initiation, the primer extension signal was markedly more intense than that in the control sample that lacked the Sarkosyl addition (Fig. 7B; compare lanes 1 and 5). Addition of Sarkosyl 3 min or later after the start of initiation, however, failed to cause an increase in the primer extension signal (Fig. 7B; compare lanes 2 to 4 with lanes 6 to 8). These results indicate that there is a short period of time when the paused polymerase is capable of resuming elongation when treated with Sarkosyl. This capacity is lost over a short period of time.

RNA polymerase II pauses in the absence of the CTD. A current model for why RNA polymerase pauses in the promoter-proximal position posits that the dephosphorylated form of the CTD could function as a tether between the RNA poly-

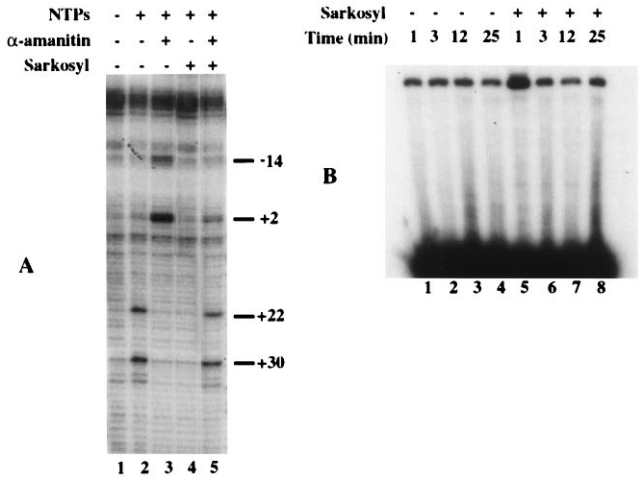

FIG. 7. Sarkosyl release of paused polymerase. (A) Permanganate analysis was performed on the −194/+84 construct. Incubation started with templates, extract, and NTPs in all samples except that no NTPs were present in lane 1. Alpha-amanitin (4 μg/ml) was added in lane 3 at the beginning of the incubation; 29 min after incubation started, 4 μg of alpha-amanitin per ml was added to lane 5; 30 min after incubation started, 0.1% Sarkosyl was added to lanes 4 and lane 5; 31 min after incubation started, all samples were treated with permanganate and processed as described in the text. (B) Time course analysis to monitor the capacity of paused polymerase to resume elongation upon release by Sarkosyl. Preinitiation complexes were assembled by incubating the template in extract for 30 min in the absence of NTPs. Transcription was initiated at time zero by adding NTPs. At various times after addition of NTPs, one aliquot was added to Sarkosyl to give a final Sarkosyl concentration of 0.1% and another aliquot was added to water. Transcription was halted 1 min after addition of each aliquot to Sarkosyl or water. Transcripts were analyzed by primer extension using the +71/+43 primer.

merase and TBP anchored at the TATA box (14, 19). To test this model, we took advantage of an alpha-amanitin-resistant form of *Drosophila* RNA polymerase (11). The presence of low concentrations of alpha-amanitin inhibited the ability of the RNA polymerase that was present in the extract from elongating into the paused state (Fig. 8A, lane 3). If highly purified alpha-amanitin-resistant polymerase was added to the extract in the presence of alpha-amanitin, this resistant polymerase was able to enter the paused state, as indicated by the appearance of permanganate hyperreactivity at +22 and +30 (Fig. 8A; compares lanes 3 and 4). Permanganate reactivity was also still evident near the transcription start site because alpha-amanitin-sensitive polymerase was present in the crude extract.

To determine if the CTD was required for pausing, we determined what would happen when the CTD was removed. An aliquot of the alpha-amanitin-resistant polymerase was digested with chymotrypsin to remove the CTD. Chymotrypsin cleaves at tyrosines found in the seven-amino-acid repeats composing the CTD and does not attack the other subunits of RNA polymerase. Previously, this treatment was shown to have no effect on the specific activity of the enzyme or its ability to respond to the transcriptional activator Sp1 (44, 45). Figure 8B shows that all of the largest subunit was converted to the CTD-minus form. Western blotting (immunoblotting) with an antibody specific for the CTD confirms that there was no detectable CTD left in the preparation (data not shown).

The CTD-minus and CTD-plus versions of the alpha-amanitin-resistant polymerase were tested for pausing in nuclear extract containing alpha-amanitin. As shown in Fig. 8C, the two forms of the polymerase paused at comparable levels. The reactivity at +22 is the clearest indicator that both forms of polymerase pause, since there is very little reactivity at this position in the controls (compare lane 3 with lane 4 in Fig. 8A and lanes 1 and 2 with lanes 3 and 4 in Fig. 8C). Further analysis showed that if the level of CTD-plus polymerase was reduced threefold, the level of permanganate reactivity at +22 and +30 approached that of the background in lanes 1 and 2 of Fig. 8C (data not shown). Hence, the level of contaminating CTD-plus polymerase in the CTD-minus preparation would have to have been at least 30% of the total if contaminating CTD-plus polymerase were to account for the permanganate

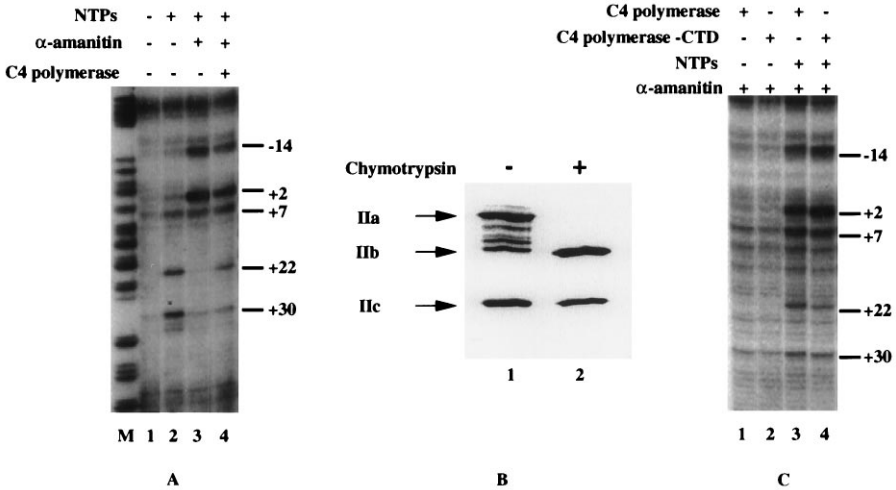

FIG. 8. CTD is not required for promoter-proximal pausing. (A) Purified alpha-amanitin-resistant polymerase pauses on the *hsp70* promoter when added to the nuclear extract. Lanes 1 to 3 show the permanganate patterns of the alpha-amanitin-sensitive polymerase endogenous to the extract. Lane 4 shows the permanganate pattern when purified alpha-amanitin-resistant C4 polymerase was added to the extract. The resistant polymerase was preincubated with the extract for 15 min, and then transcription was performed in the presence of 4 μg of alpha-amanitin per ml. (B) SDS-PAGE analysis of the intact C4 polymerase (lane 1) and chymotrypsin-treated C4 polymerase (lane 2). One hundred sixty nanograms of each preparation was analyzed, and polymerase subunits were detected by silver staining. IIa and IIb refer, respectively, to the intact and proteolyzed forms of the largest subunit of polymerase. Some proteolysis of the IIa form occurs during purification of the polymerase. IIc designates the second largest subunit of the polymerase. Smaller subunits of the polymerase are not shown. (C) Pausing of polymerase lacking the CTD. *hsp70* promoter DNA was incubated with extract that had been preincubated with intact (lanes 1 and 3) and proteolyzed (lanes 2 and 4) forms of the C4 polymerase. All reaction mixtures contained alpha-amanitin (4 μg/ml) to inhibit the sensitive polymerase that is endogenous to the extract.

reactivity observed in lane 4. Since the level of contamination was undetectable (Fig. 8B, lane 2), we conclude that the CTD can be removed without significantly affecting the ability of RNA polymerase II to pause on the *hsp70* promoter in vitro.

DISCUSSION

We have shown that RNA polymerase pauses on the *hsp70* and histone H3 promoters in a nuclear extract derived from *Drosophila* embryos. Analysis of the transcripts produced from the *hsp70* promoter indicates that more than 50% of the polymerases that initiate fail to elongate beyond 40 nucleotides. This primer extension analysis has been repeated numerous times, and the level of polymerase that fails to elongate beyond the promoter-proximal region ranges between 50 to 70%. The permanganate assay is a better assay for detecting promoter-proximal pausing. It directly monitors template-engaged polymerases. Moreover, it is more sensitive than the primer extension analysis because the permanganate reactivity at certain positions such as +22 is extremely low in the absence of paused polymerase.

Several observations indicate that the pausing detected in our cell-free system using permanganate is directly related to the pausing that occurs in vivo. First, the pattern of permanganate reactivity observed in vitro is strikingly similar to the pattern observed on the promoters in nuclei derived from embryos containing these promoters constructs. Moreover, the pattern of permanganate reactivity observed on the *hsp70* promoter matches well with the pattern observed on the endogenous *hsp70* promoter in intact, cultured *Drosophila* cells (8). Small variations between the patterns observed in vitro and in vivo could reflect heterogeneity in the exact positions where polymerases pause. Rasmussen and Lis mapped the 3′ terminus of the nascent RNA associated with paused polymerase and deduced that polymerases pause throughout the region from +20 to +35 (27, 28). Prominent sites of pausing occur at +20 and +30. The finding that polymerase paused on the histone H3 promoter in the reconstituted system was initially a matter of concern because paused polymerase had not been detected on the endogenous histone H3 promoter (40). The endogenous histone genes are present in multiple copies (18). When a single copy of the H3 promoter that was introduced on a P element was analyzed, paused polymerase was evident. It is likely that heterogeneity in the activity of the endogenous H3 promoters obscures detection of the paused polymerase (9).

In addition to the similarity in the patterns of permanganate reactivity, pausing on *hsp70* in vitro and pausing on *hsp70* in vivo have similar sequence requirements. Deletion of sequences between −89 and −50 leads to a substantial decrease in the level of permanganate reactivity. This deletion removes a GAGA element that has been shown to contribute to the level of paused polymerase in vivo (16). The low level of paused polymerase that is still associated with the −50 deletion may be due to several low-affinity binding sites for the GAGA factor in the region between −50 and the transcription start site (references 10 and 24 and unpublished results). Since the deletion of the region between −89 and −50 also causes a decrease in the level of permanganate reactivity found at the transcription start when alpha-amanitin is present, it appears that the region between −89 and −50 contributes primarily to the recruitment of RNA polymerase II rather than to the efficiency of pausing. This would be consistent with the finding that GAGA factor can increase the accessibility of DNA in reconstituted chromatin (34, 37). It seems most likely that the effect of our deletion is due to the loss of a GAGA element. However, the region interacts with both heat shock factor (42) and TFIID (26), and so more analyses are needed to definitively establish the contribution of the GAGA factor.

We have also found by analyzing a series of 3′ deletions that specific sequences are not required downstream from +33 to reconstitute pausing. This is in good agreement with what has been observed in vivo (16). Our results help emphasize the point that pausing is not likely to arise from a barrier generated by the association of proteins with specific sequences in the region downstream from the pause site. Deletion of the region between +33 and +23 results in a decrease in the level of paused polymerase in vitro. On the basis of the observation that the level of permanganate reactivity near the transcription start in the presence of alpha-amanitin is also reduced by the deletion, it appears that our sequence changes once again affected the recruitment of the polymerase rather than the efficiency of pausing. TFIID recognizes sequences in the region between +33 and the transcription start in *hsp70* (26, 36). Hence, the reduction in the level of polymerase recruited to the promoter is probably due to an inhibition of TFIID binding.

Establishing a strong correlation between the pausing that occurs in vitro and in vivo now opens the process of pausing up for biochemical analysis. We have begun to investigate mechanistic aspects of the pausing process. First, we determined if the paused polymerase is a static or dynamic situation in the reconstituted reaction. This would be pertinent to understanding the mechanism by which an activator might work. If paused polymerase is a stable complex, activation would need to act directly on the paused polymerase. On the other hand, if there were continuous rounds of initiation and premature termination, the activator could function by recruiting a version of polymerase that overcomes the pause. All of the available in vivo results are essentially snapshots of the cell and cannot distinguish between a static and a dynamic situation. Analysis of the cell-free system indicates that the polymerase enters the paused state within 1 min of initiation and persists in this state for at least 25 min. The observation that KCl or detergent must be added to nuclei in order to release the paused polymerase in a nuclear run-on suggests that the paused polymerase in cells is very stable (29). Together with our in vitro analysis, the data support a model whereby the polymerase is stably bound in the paused position, waiting for some signal that causes its release. Even if there is a slow rate of release and replacement on the inactive *hsp70* promoter, the rate of this exchange is far too slow to accommodate the rapid rate at which these genes are induced by heat shock. Full induction of the genes is observed within 60 s of heat shock (23).

Further analysis of the paused polymerase that we have reconstituted indicates that it is initially capable of resuming elongation when Sarkosyl is added, but this capacity is rapidly lost. Marshall and Price (20) also observed that much of the polymerase initiating in their reactions appears to pause in a promoter-proximal region and that these polymerases retain the ability to resume elongation when treated with Sarkosyl or KCl for a period of only a few minutes. Both of these results point to some process whereby the polymerase loses its ability to resume elongation when released with KCl or Sarkosyl.

Our results shed new light on data recently reported by Rasmussen and Lis (28). They have analyzed the short transcripts from numerous genes, including *hsp70*, that copurify with nuclei. Two types of transcripts were found that have 3′ ends mapping within the first 50 nucleotides of the transcription start. One type of transcript must be associated with transcriptionally engaged polymerases, since this type was extended when NTPs and Sarkosyl were added to the nuclei. A second type of transcript was not extended when Sarkosyl and

NTPs were added. In the case of *hsp70*, the first type (extendable) was more prevalent than the latter, whereas the opposite was the case for the *hsp26* and *hsp27* heat shock genes. It was suggested that the transcripts that failed to be extended could be products of premature termination. By definition, premature termination implies that the transcription complex has disengaged from the template. Our results indicate that the nonextenable transcripts might still be associated with polymerases that are engaged on the template.

The basis for transcriptionally competent and incompetent polymerase remains to be determined. Obviously, KCl and Sarkosyl are not natural activators of transcription. The finding that some polymerases are incapable of elongation after addition of these agents may not be pertinent to what actually happens in the cell, but it clearly points to the existence of biochemical modifications that need to be investigated further.

The mechanism of promoter-proximal pausing is not understood. One appealing model that has recently emerged is one whereby the CTD serves as a tether between the RNA polymerase and a molecule of TBP that would be anchored on the TATA element (14, 19). The model stems from the observations that the dephosphorylated form of the CTD binds TBP (35) and that the CTD of the paused polymerase in vivo is primarily in the dephosphorylated state (22, 41). The cell-free system has provided us with the first opportunity to evaluate this model critically. Our results demonstrate that removal of the CTD does not significantly inhibit the ability of RNA polymerase II to pause. This finding suggests that the CTD is not involved in pausing RNA polymerase on the *hsp70* promoter. It is important to note, however, that our experiment does not address whether the CTD is involved in the release of the polymerase.

Other interactions that form during assembly of the initiation complex might form the tether (43). We find that TFIID makes contact with the *hsp70* promoter as far as 45 nucleotides downstream from the transcription start site, raising the possibility that TFIID somehow contributes to pausing (33). Whatever the mechanism of pausing, it must take into account that pausing is still detected even when the heat shock gene is fully induced (8). Further analysis of pausing by fractionating our cell-free system could provide the information needed to understand the basis for promoter-proximal pausing.

The extract that we have used has been prepared by a widely used method (2). We have also detected paused polymerase on the *hsp70* promoter in extracts prepared in a slightly different way from KcH cells, a *Drosophila* tissue culture line (data not shown; see reference 25 for extract preparation). For both extracts, the permanganate hyperreactivity that is associated with the paused polymerase is evident only when a low level of promoter DNA is used. We suspect that our conditions allow RNA polymerase to be loaded onto a substantial fraction of the promoters present in the extract. As a result, the permanganate signal from the paused polymerase readily exceeds the background from nontranscribed templates. Interestingly, the activity of transcriptional activators in these crude extracts is most apparent under conditions in which DNA concentrations are kept low relative to the amount of extract (5, 13, 32). Our results should alert investigators to the possibility that transcriptional activation mechanisms reconstituted in vitro with *Drosophila* extracts may act at the level of transcriptional elongation rather than initiation. This should no longer come as a surprise, since Lis and colleagues (8, 28, 30) have provided evidence for pausing on the majority of promoters that they have analyzed in vivo. Moreover, the combination of the results presented here and the observations of Marshall and Price (20) provide evidence that pausing occurs in vitro on at least four unrelated *Drosophila* promoters.

ACKNOWLEDGMENTS

We thank Quinn Lu for transforming the −50 deletion into flies while he was in Sarah Elgin's laboratory. We thank David Price for providing the KcH nuclear extract that was tested for pausing.

This work was supported by NIH grants GM47477 to D.S.G. and GM28078 to A.L.G.

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