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# RNA Polymerase II Pauses at the 5′ End of the Transcriptionally Induced *Drosophila hsp70* Gene

THOMAS O'BRIEN[1] AND JOHN T. LIS[2]*

*Section of Genetics and Development[1] and Section of Biochemistry, Molecular and Cell Biology,[2] Cornell University, Ithaca, New York 14853*

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**An RNA polymerase II molecule is associated with the 5′ end of the *Drosophila melanogaster hsp70* gene under non-heat shock conditions. This polymerase is engaged in transcription but has paused, or arrested, after synthesizing about 25 nucleotides (A. E. Rougvie and J. T. Lis, Cell 54:795–804, 1988). Resumption of elongation by this paused polymerase appears to be the rate-limiting step in *hsp70* transcription in uninduced cells. Here we report results of nuclear run-on assays that measure the distribution of elongating and paused RNA polymerase molecules on the *hsp70* gene in induced cells. Pausing of polymerase was detected at the 5′ end of *hsp70* in cells exposed to the intermediate heat shock temperatures of 27 and 30°C. At 30°C, each copy of *hsp70* was transcribed approximately five times during the 25-min heat shock that we used. Therefore, once the *hsp70* gene is induced to an intermediate level, initiation of transcription by RNA polymerase II remains more rapid than the resumption of elongation by a paused polymerase molecule.**

Procaryotic and eucaryotic organisms frequently regulate gene expression at the level of transcription, which itself can be controlled at many different points. Potential control points include the formation of a preinitiation complex, the initiation of transcription, or the elongation of the transcriptional complex. Control of elongation as a way of modulating gene expression has been found to occur for several well-characterized procaryotic genes (for a review, see reference 24). In eucaryotes, examples of control at the level of elongation have also been identified; these include transcription of the human immunodeficiency virus type 1 long terminal repeat (25), the simian virus 40 late transcription unit (1), and the major late transcription unit of adenovirus (18) and of the c-*myc* (3, 40), c-*myb* (2), c-*fos* (8), human adenosine deaminase (4, 17), and *Drosophila melanogaster hsp70* (27) genes (for a review, see reference 31).

In the case of the *D. melanogaster hsp70* gene, elongation control occurs very close to the site of transcriptional initiation. In vivo UV cross-linking studies revealed that a molecule of RNA polymerase II is associated with DNA sequences near the transcription start site in cells that are not heat shock induced (10). The transcriptional competency of this polymerase molecule was also examined by nuclear run-on assays (27). These studies revealed that in uninduced cells, RNA polymerase has access to the *hsp70* promoter and can initiate transcription. However, this polymerase pauses at approximately position +25, and without further stimulus it does not normally proceed efficiently beyond this point. The presence of a paused RNA polymerase molecule on an uninduced *hsp70* gene may serve as a mechanism for rapidly providing a round of transcription in response to heat shock. Additionally, the presence of this paused polymerase identifies a rate-limiting step in transcription control in uninduced cells. Here, we show that pausing of RNA polymerase II on the *hsp70* gene is not restricted to uninduced cells. At the intermediate heat shock temperature of 30°C, pausing of polymerase can still be detected at the 5′ end of the *hsp70* gene. This finding indicates that at low levels of induction, RNA polymerase initiates transcription more rapidly on the *hsp70* promoter than it elongates from the pause. Therefore, under these conditions, elongation of polymerase from this pause remains the rate-limiting step in transcription.

## MATERIALS AND METHODS

**Plasmids.** Bacterial clones containing the core histone genes of *D. melanogaster*, *H2A*, *H2B*, *H3*, and *H4*, were generated by subcloning fragments of cDm500 (15) into the *Sma*I site of pUC19 (13). The subcloned fragments from the 4.8-kb histone repeat unit were *Apa*I-*Stu*I for *H2A*, *Bgl*II-*Apa*I for *H2B*, *Ava*I-*Hpa*I for *H3*, and *Stu*I-*Ava*I for *H4*. The *H4* gene was further subcloned by removing a *Dra*I-*Hin*dIII fragment containing the entire histone gene and placing into pUC19 digested with *Sma*I-*Hin*dIII. The resulting plasmid DNAs were each digested with the restriction enzymes *Eco*RI and *Hin*dIII to give a single fragment containing an entire histone gene.

Plasmid p70X2.6, containing the entire *hsp70* transcription coding region cloned into the *Xho*I linker of pUC13X (27), was digested with *Ban*I and *Sca*I. The predicted *Ban*I site at +1901, with respect to the transcription initiation site of *hsp70* (36), was not cleaved in the copy of the *hsp70* gene used in this study. Also, the *Ban*I site at position 216 in pUC13X was not found to cut in these digestions. The consensus sequence for *Ban*I is GGPyPuCC (33), both of the sites that are not cut have the sequence GGCGCC, and the sites that are cleaved have the sequence GGTGCC or GGCACC. *Nar*I cleaves the sequence GGCGCC, and it was used to confirm the existence of these sequences in p70X2.6. Perhaps *Ban*I does not cleave this sequence efficiently, or the sequence is modified in a way that prevents *Ban*I, but not *Nar*I, from cutting.

Approximately 3 μg of each digested plasmid was electrophoresed on 1% agarose gels and transferred to a GeneScreen Plus membrane as described previously (27).

**Nuclear run-on assays.** *Drosophila* Schneider line 2 (SL2) cells were shifted from growth at 23°C to various heat shock temperatures (27, 30, 33, and 36.5°C) for 25 min by circulat-

* Corresponding author.

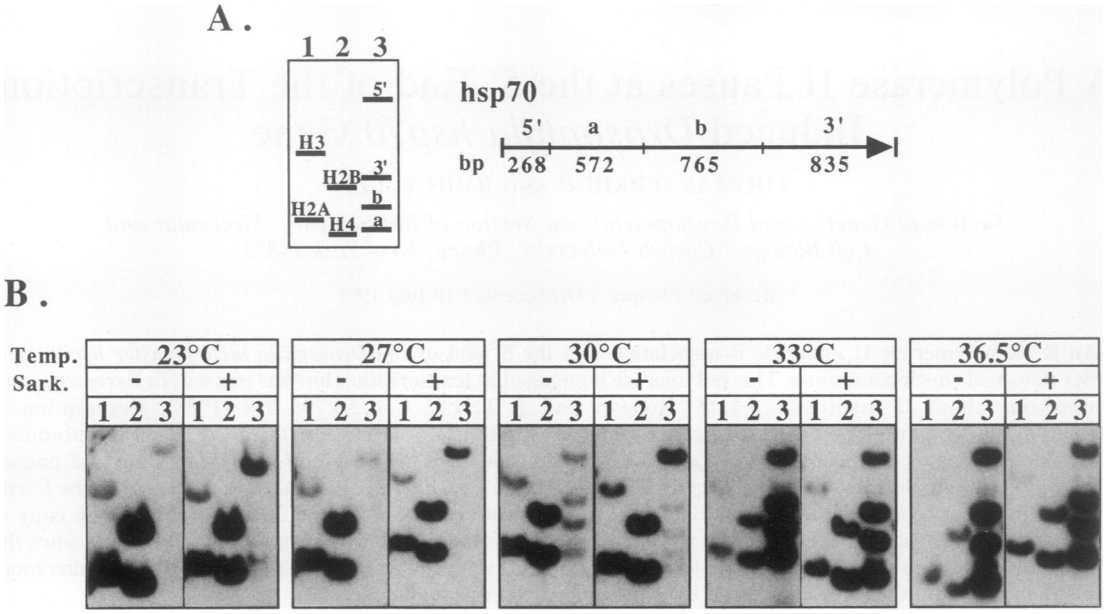


FIG. 1. Temperature induction of the *Drosophila hsp70* gene. (A) Key to Southern blots. Transcription coding regions from each of the core histones were included on each filter as an internal standard. Plasmids containing the histone genes were digested with the restriction enzymes *Eco*RI and *Hin*dIII to give a single fragment containing an entire histone gene (lanes 1 and 2). In the experiment shown, digested plasmids containing the *H2A* and *H3* genes and the *H2B* and *H4* genes were loaded in the same lanes. Sizes of the fragments are 594 bp for *H2A*, 815 bp for *H2B*, 1,100 bp for *H3*, and 466 bp for *H4*. Plasmid p70X2.6, which contains the entire *hsp70* gene (27), was digested with *Ban*I and *Sca*I to give the indicated fragments (lane 3). The numbers in base pairs indicate the size of each fragment that is homologous to the *hsp70* RNA; heavy vertical lines indicate the regions of transcription initiation and termination, whereas the lighter lines indicate restriction enzyme digestion sites. The sizes of these fragments are 1,437 bp for the 5′ fragment (268 bp of transcription unit and the rest plasmid vector sequence), 572 bp for fragment a, 765 bp for fragment b, and 974 bp for the 3′ fragment (835 bp of transcription unit and the rest plasmid vector sequence). (B) Nuclear run-on assays in the presence or absence of 0.6% sarcosyl. *Drosophila* SL2 cells were heat shocked at the indicated temperatures for 25 min, and nuclei were isolated as described previously (27). The transcription assay was performed as described previously (28) in the presence (+) or absence (−) of 0.6% sarcosyl, except that it was allowed to continue for only 2 min. The bands seen in some of the *hsp70* lanes that are not of the indicated sizes are likely due to *Ban*I cutting inefficiently at two of its recognition sequences (see Materials and Methods). As the levels of RNA hybridizing to these fragments are very low relative to the overall levels, they should not significantly affect our conclusions.

ing water through a jacketed spinner flask. Nuclei were isolated, and run-on assays were carried out at room temperature (22 to 23°C) in the presence of [$^{32}$P]UTP as previously described (28). Radiolabeled RNA was preincubated at 42°C to a blank GeneScreen Plus hybridization transfer membrane for at least 6 h (to reduce background) and then hybridized to GeneScreen Plus filters containing restriction endonuclease-cut DNA on a Wheaton roller bottle apparatus in a total volume of 5 ml of hybridization buffer (27). Filters were analyzed either by autoradiography and densitometry (with a Bio-Rad model 620 video densitometer) or by direct detection on a Betagen Betascope 603 blot analyzer. Appropriate exposures of Kodak XAR-5 film were used for densitometry to ensure that the data collection was within the linear range.

**Indirect immunofluorescence of HSP70 protein in SL2 cells.** Cells were heat shocked at each temperature as described above for 1 h. HSP70 protein levels in individual cells was determined by using an HSP70-specific monoclonal antibody in conjunction with a fluorescein-labeled secondary antibody (37). A Nikon Diaphot inverted microscope with a 40× Fluor objective was used to examine fluorescence intensity per cell. Images were acquired with a microchannel plate image intensifier (Video Scope, Int.) coupled to a silicon diode video camera (Dage-MTI). Neutral density filters were used as required to ensure that the data collection was within the linear range of detection. The signal was then passed into a Tracor Northern TN-8500 image analysis system, and the fluorescence intensity of individual cells was determined. Photographs were taken with a Zeiss Universal fluorescence microscope and a 25× Plan Neofluor lens and with Kodak TMax p3200 film. All exposures were for 8 s.

## RESULTS

**A paused RNA polymerase II molecule is detected at the 5′ end of *hsp70* when transcribed at intermediate levels.** Figure 1A shows the pattern of DNA fragments of *hsp70* expected after digestion of plasmid sequences with the restriction enzymes *Ban*I and *Sca*I. The digests divide the transcription region into four fragments. Each filter also contains the transcription units of the *Drosophila* core histones *H2A*, *H2B*, *H3*, and *H4*. These are included as standards to allow *hsp70* signals from different heat shock conditions to be directly compared. The levels of transcription of the core histones do not vary dramatically between non-heat shock and full heat shock temperatures (7, 9), and we assume that their level of transcription also remains relatively constant at intermediate heat shock temperatures.

*Drosophila* SL2 cells were exposed to various temperatures for 25 min, nuclei were isolated, and RNA polymerase II distributions on the *hsp70* and core histone genes were assayed by in vitro nuclear run-on assays. Figure 1B shows the hybridization of labeled run-on transcripts to the histone

genes and to restriction fragments of the *hsp70* gene. At 23°C, very low levels of labeled RNA homologous to the *hsp70* transcription regions are detected. However, as previously observed (27), if 0.6% sarcosyl (an anionic detergent) is included in the nuclear run-on reaction, a much higher level of radiolabeled RNA homologous to the 5′ end of the *hsp70* gene is detected. This concentration of sarcosyl strips many chromosomal proteins from DNA (29), but transcriptionally engaged RNA polymerases are left fully competent for elongation (11, 27). Moreover, this treatment prevents new initiations by RNA polymerase II (11, 27). Therefore, sarcosyl or high salt concentrations (27) release a polymerase molecule into the body of the gene that is transcriptionally engaged but normally paused at the 5′ end of the uninduced *hsp70* gene.

Transcription by the sarcosyl-released polymerase in uninduced cells is detected only on the 5′ fragment. A short run-on time ensures that the released polymerase does not transcribe farther than 268 bp, which is the length of the RNA homologous region on the 5′ fragment (Fig. 1A). Stimulation of transcription by sarcosyl at the 5′ end of the gene is also seen at 27 and 30°C (Fig. 1B). Thus, at these temperatures, the 5′ end of the *hsp70* gene contains a paused polymerase that can elongate in the presence of sarcosyl. Although at 33 and 36.5°C sarcosyl does not obviously alter the ratio of run-on RNAs homologous to the 5′ fragment relative to those homologous to fragments in the body of the *hsp70* gene, the high level of elongating polymerase makes it difficult to detect an additional, sarcosyl-released polymerase (Fig. 1B).

**Estimation of the number of paused and elongating RNA polymerase II molecules on *hsp70* at intermediate heat shock temperatures.** The number of RNA polymerase II molecules on a fully induced *hsp70* gene has been previously estimated by two independent techniques. First, the rates of synthesis of *hsp70* mRNA in *Drosophila* SL2 cells and embryos were estimated to be 10 and 30 molecules per min, respectively (R. Freund, quoted in reference 6; 14), with the average of these estimates being 20 mRNA molecules per min. As the elongation rate of RNA polymerase II in *D. melanogaster* is about 1.1 kb/min at 25°C (35), and the length of the *hsp70* transcript is 2.4 kb, then approximately 40 molecules of RNA polymerase II are on the fully induced *hsp70* gene (assuming that the rate of movement of polymerase in vivo at 36.5°C is similar to that at 25°C). Second, direct examination of the number of growing RNA chains in Miller spreads (19, 20) reveals an average number of 22 polymerase molecules per *hsp70* gene (22). The average of these two estimates is approximately 30 polymerase molecules per gene.

Using this value of 30 RNA polymerase molecules per gene for the fully induced (36.5°C) *hsp70* gene, we estimated the number of polymerase molecules on each restriction fragment of the *hsp70* gene at the lower temperatures (Fig. 2). The total number of elongating polymerases on the *hsp70* gene at each temperature was calculated from nuclear run-on assays carried out in the absence of sarcosyl (Table 1). At 30°C, the number of elongating polymerases is clearly greater than in the uninduced control, indicating that these cells are responding to the mild heat shock. A further increase is seen at 33°C; however, there is a large standard deviation associated with this value, presumably because this temperature is in the steepest region of the thermal induction curve. At this temperature, slight variations in heat shock temperatures could be expected to give large variations in levels of polymerase. The overall profile of heat shock induction is similar to that of previous studies that examined the levels of *hsp70* mRNA and HSP70 protein at different heat shock temperatures (16, 32).

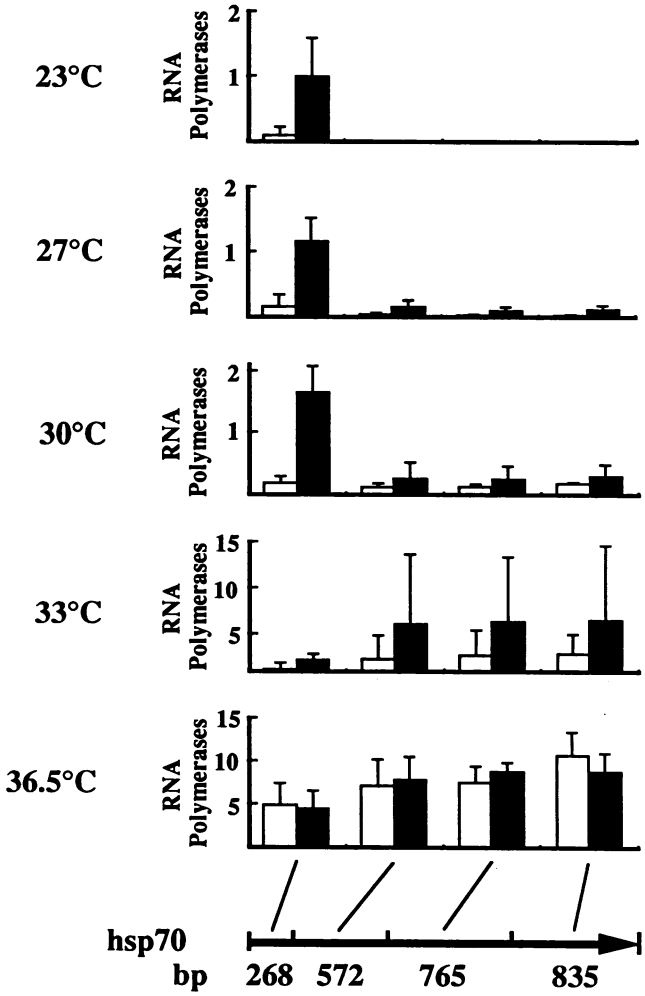


FIG. 2. Number of RNA polymerase II molecules on *hsp70* at each heat shock temperature. Data are averages of four separate experiments, and the standard deviation associated with each average is shown. The level of hybridization to each restriction fragment was determined by densitometry (different exposures of autoradiograms were used to ensure that all bands were scanned within the linear range of detection) or by direct detection on the Betagen Betascope 603 blot analyzer. The histone genes were used as an internal standard to allow direct comparison of transcription levels on the *hsp70* gene at different temperatures. The numbers were calculated as follows. Each fully induced (36.5°C) *hsp70* gene is assumed to have 30 transcribing polymerase molecules (see text). To minimize any effects that sarcosyl may have on transcription, polymerase numbers for minus- and plus-sarcosyl filters were calculated separately. As the nuclei used in the 36.5°C sample are identical except for the presence or absence of 0.6% sarcosyl in the run-on assay, we assume there are 30 transcribing polymerase molecules per gene in each case. With this number, and using the amount of RNA on the core histone genes as a standard, we calculated the number of polymerase molecules on the *hsp70* gene at each temperature. Filled bars indicate the total number of polymerase molecules calculated on each fragment when the nuclear run-on assay is carried out in the presence of 0.6% sarcosyl, and open bars are the numbers calculated in run-on assays lacking sarcosyl.

At 23, 27, and 30°C, more RNA was transcribed from the 5′ fragment when sarcosyl was present in the nuclear run-on

TABLE 1. Number of elongating and paused RNA polymerase II molecules on the *hsp70* gene at different heat shock temperatures[a]

| Temp (°C) of heat shock | No. of elongating polymerase molecules | No. of paused polymerase molecules |
|---|---|---|
| 23 | 0.1 ± 0.1 | 0.9 ± 0.6 |
| 27 | 0.2 ± 0.2 | 1.0 ± 0.2 |
| 30 | 0.6 ± 0.02 | 1.5 ± 0.4 |
| 33 | 9.0 ± 7.9 | ND |
| 36.5 | 30 | ND |

[a] Values are averages of four independent experiments. The number of elongating RNA polymerase molecules was calculated from Fig. 2. The numbers of polymerase molecules present on the individual restriction fragments of *hsp70* with nuclear run-on assays carried out in the absence of sarcosyl were added to give a total number of polymerase molecules per gene. The number of paused RNA polymerase molecules per gene was calculated by subtracting the number of polymerase molecules on the 5′ fragment in the absence of sarcosyl from the number calculated from run-on assays in the presence of sarcosyl. The averages for 23, 27, and 30°C, with the standard deviations, are shown. We were unable to determine whether there was any paused polymerase at 33 or 36.5°C. ND, could not be determined.

assay than when sarcosyl was absent (Fig. 2). The number of paused polymerases is the difference between the number of transcribing RNA polymerases on the 5′ fragment in the absence and in the presence of sarcosyl. When *hsp70* is not induced, there is approximately 0.9 of a RNA polymerase molecule per gene paused near the promoter (Table 1). UV cross-linking of RNA polymerase II to the uninduced *hsp70* 5′ region had previously estimated this number to be about 1 (10), but this technique is unable to differentiate between transcriptionally competent and incompetent polymerases. Our quantative run-on analysis indicates that most of the polymerase at the promoter region of *hsp70* is transcriptionally competent. At 27 and 30°C, this high level of paused polymerase persists (Table 1).

**The heat shock response at intermediate temperatures is fairly homogeneous.** At low levels of induction, elongation of polymerase from the pause remains slower than initiation of transcription on the *hsp70* gene. This conclusion, however, is dependent upon the cell population responding to the heat shock stimulus in a fairly homogeneous manner. Since each genome contains five copies of the *hsp70* gene (12) and SL2 cells have a tetraploid karyotype (30), the response of each copy within the cell to the heat shock stimulus may vary. It is not possible to examine the transcriptional activity of *hsp70* genes on each individual chromatid, but it has been demonstrated by in vivo UV cross-linking that the five genomic copies of *hsp70* are expressed at similar levels upon heat shock (10). It is also possible that the majority of cells remain uninduced at 30°C and the level of 0.6 of a transcribing polymerase molecule detected (Table 1) represents high activity from a small fraction of the cells. In this scenario, the paused polymerase could simply be contributed by cells that remain uninduced.

To examine the response of individual cells to the heat shock in the SL2 cell population, we determined the level of expression of the heat shock protein, HSP70, in individual cells by indirect immunofluorescence with antibody specific for HSP70 (37). The immunofluorescence intensity of cells was determined as described in Materials and Methods. We first used phase-contrast images to identify cells on the basis of size and morphology, and this image was then overlaid upon the immunofluorescence image to define each area for fluorescence measurement. The average level of HSP70 protein per cell at 30°C was higher than the levels detected in cells incubated at either 23 or 27°C; moreover, at 30°C, only

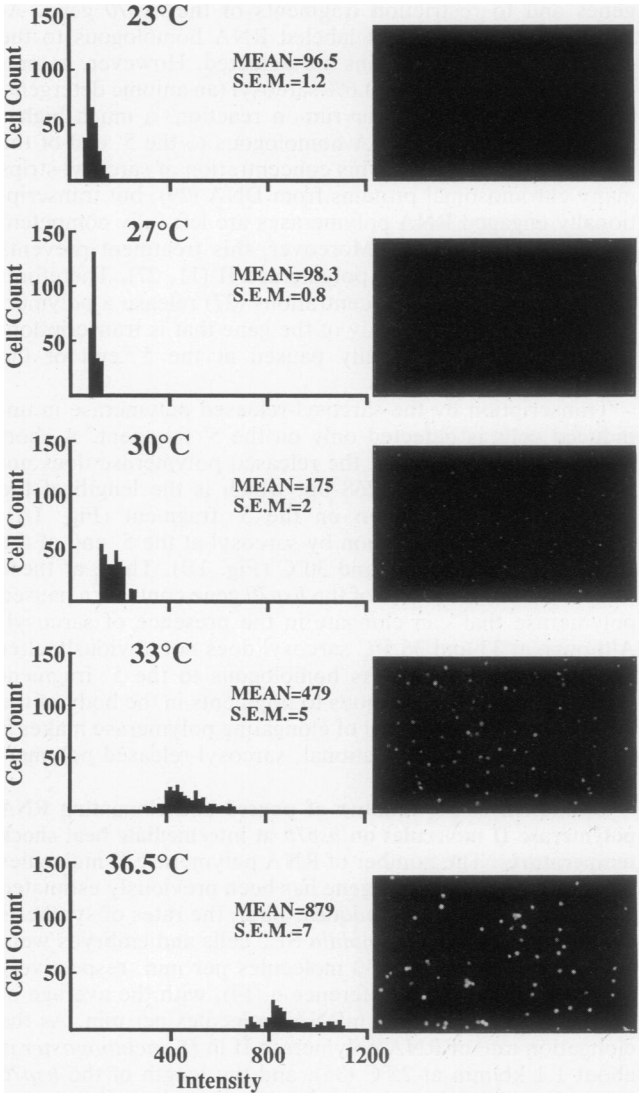


FIG. 3. HSP70 protein levels in *Drosophila* SL2 cells at different heat shock temperatures. Cells were heat shocked in a spinner flask at the indicated temperature for 1 h, and HSP70 protein levels were examined by using an HSP70-specific antibody (37). The immunofluorescence intensity values for each cell were calculated on a Tracor Northern TN-8500 image analysis system as described in Materials and Methods. Frequency distributions of these intensities for each temperature are shown, along with the mean and standard error of the mean (S.E.M.). An immunofluorescence photographs of an example of each cell population is also shown. Each exposure was manually set at 8 s.

20% of the cells had levels of HSP70 protein within 2 standard deviations of the range of the 23°C sample (Fig. 3). If the only cells that contained a paused polymerase at 30°C were the 20% that fell within the 23°C range, then we would expect to see a large decrease (fivefold) in the level of paused polymerase at 30°C relative to 23°C. Instead, the level in both cases is approximately 1 (Table 1). Also, at 30°C, all cells had HSP70 protein levels lower than those found in cells incubated at 33°C (Fig. 3), indicating that there is no small subpopulation of cells that is expressing the *hsp70* gene at a high level. Therefore, the response to heat shock appears to be fairly uniform among cells at 30°C. The

difference in levels of HSP70 protein at 23 and 30°C is unlikely to be due to large differences of message stability or differential rates of translation, as the production of a modified *hsp70* protein at 33°C is similar to that at 25°C (23). Our results therefore demonstrate that the SL2 cell population responds in a relatively homogeneous manner to a 30°C heat shock and that most of the cells have transcribed the *hsp70* gene.

After 25 min at 30°C, each *hsp70* gene has, on average, 0.6 elongating polymerase molecule (Table 1). Since the rate of polymerase elongation is about 1.1 kb/min at 25°C (35), and it takes 5 min for cells to reach the heat shock temperature, then at 30°C each *hsp70* gene has been transcribed approximately 5.5 times during the period of the heat shock. Therefore, since each gene has undergone multiple rounds of transcription, the bulk of the paused polymerase at the 5′ end must have initiated transcription during the period of the heat shock.

## DISCUSSION

The aim of this study was to determine whether RNA polymerase II continued to pause at the 5′ end of the *Drosophila hsp70* gene once the gene became transcriptionally induced. We found that at 27 and 30°C, pausing of polymerase could still be detected at the 5′ end of *hsp70* (Fig. 1; Table 1). As the cell population responded to the 30°C heat shock in a fairly homogeneous manner (Fig. 3), we estimate that each *hsp70* gene was transcribed approximately five times during the period of heat shock. Therefore, elongation of RNA polymerase II from the pause remains rate limiting in *hsp70* transcription, at least at low levels of induction.

How does heat shock increase the rate of transcription of *hsp70*? When the *hsp70* gene is uninduced, RNA polymerase can initiate transcription but pauses about 25 nucleotides from the start site (27). The rate of polymerase release from the pause into the body of the gene must be higher at 30 than at 23°C since there is a higher level of elongating polymerase at 30°C but a similar level of paused polymerase at both temperatures (Table 1). It has been demonstrated that upon heat shock a transcription factor, heat shock factor (HSF), binds upstream of heat shock genes to modulate transcription levels (5, 34, 38, 39). HSF could increase the rate of transcription in one of two ways. First, it could interact (either directly or indirectly) with the paused polymerase complex to stimulate elongation. A new polymerase could then be recruited to the promoter once the pause site becomes vacant. Alternatively, HSF may recruit new polymerase to the promoter region. The recruited polymerase then displaces the paused polymerase, forcing it into an elongation mode.

Not all transcribing polymerases necessarily have an equivalent elongation competence. We (and an alert reviewer) have noticed that the ratio of transcription on the body of the *hsp70* gene to that on the histone genes is higher (ca. threefold on average for all experiments) in the absence than in the presence of sarcosyl (Fig. 1B, 36.5°C sample). Perhaps this reflects a higher elongation competence of RNA polymerases on induced *hsp70* genes than on histone genes in intact nuclei which have not been disrupted by detergent or high-salt treatments. Polymerases that are modified to escape the pause may be endowed with properties that allow them to elongate very efficiently throughout the gene.

We were unable to determine whether polymerase pauses at the 5′ end of *hsp70* at higher induction temperatures (33 and 36.5°C). This may be due to the difficulty in detecting the signal contributed by the sarcosyl-stimulated elongation of a paused polymerase over the high level of transcription elongation occurring on the gene. It is also possible that at high levels of heat shock induction release of polymerase from the pause is no longer rate limiting, and therefore polymerase would not accumulate at the pause site.

We have shown here that at low levels of transcriptional induction, initiation of transcription at the 5′ end of the *Drosophila hsp70* gene can occur more rapidly than release of paused polymerase into the body of the gene. This finding demonstrates that under these conditions, elongation of polymerase from the pause remains the slowest step in transcription of *hsp70*. Pausing of polymerase can also be detected at the 5′ end of other *Drosophila* heat shock genes, such as *hsp26* (28), *hsp83* (26), and *hsp23* and *hsp27* (21). Also, many constitutively expressed genes contain a sarcosyl-releasable polymerase at their 5′ ends. These genes include those encoding β1-tubulin, glyceraldehyde phosphate dehydrogenases 1 and 2, and polyubiquitin (28). However, for these genes, it has not yet been demonstrated whether each copy that contains a paused polymerase also contains an elongating polymerase, which we have found to occur on the *hsp70* gene at 30°C. Nevertheless, elongation of RNA polymerase II from a paused configuration may be rate limiting for many *Drosophila* genes and therefore a general target for transcriptional modulation.

## ACKNOWLEDGMENTS

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