Transcription of Heat Shock Loci of *Drosophila* in a Nuclear System†

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ABSTRACT: We have investigated transcription in nuclei isolated from 6–18-h *Drosophila melanogaster* embryos. Kinetic aspects and specific products of the reaction have been analyzed; in particular, we have examined whether or not a faithful representation of an induced state, in this case the heat shock response, is maintained in the isolated nuclei. RNA polymerase II is active for at least 30 min. The combined RNA polymerase I and III activities are seen to continue synthesis for 60 min. Maximal synthesis by all enzymes is obtained in the absence of Mn^{2+}. Transcripts from the 5S DNA have been characterized. These consist of two species, of 135 and 120 nucleotides, apparently the precursor and mature forms of 5S RNA. Extensive initiation by RNA polymerase III occurs on 5S DNA in this system. Transcription from the heat shock loci at 87A and 87Cl has been analyzed by hybridization of the newly synthesized RNA (selected by using 5′-mercuri-UTP) to plasmids containing the *hsp*70 gene and adjacent regions. Only those segments of the DNA to which transcripts have been mapped in vivo hybridized with the in vitro synthesized RNA, and this transcription was observed only in nuclei isolated from heat-shocked embryos; no transcripts are detected in nuclei isolated from control embryos. These RNA species are synthesized by RNA polymerase II. Further analysis of transcription of the *hsp*68 gene (at locus 95D) has also been carried out. We conclude that in this system RNA polymerases II and III transcribe the chromatin template accurately and that the changes related to gene activation by heat shock are stable during nuclear isolation.

In vitro transcription systems composed of isolated nuclei have proven particularly useful in the study of gene expression. These systems rely on the endogenous polymerase activities present in the nucleus at the time of isolation. Typically, all three classes of polymerase are present [e.g., see Marzluff et al. (1974) and Udvardy & Seifart (1976)]: RNA polymerase I, responsible for ribosomal RNA (rRNA) synthesis; RNA polymerase II, responsible for messenger RNA (mRNA) and heterogeneous nuclear RNA (hnRNA) synthesis; and RNA polymerase III, responsible for 5S and transfer RNA (tRNA) synthesis, as well as various small RNAs [see Roeder (1976) for a review]. In isolated nuclei, the activity of the respective enzymes can be determined because of their varying sensitivity to the inhibitor α-amanitin, with generally RNA polymerase II the most sensitive, RNA polymerase III less sensitive, and RNA polymerase I insensitive. In several instances, the RNA polymerases in isolated nuclei have been shown to transcribe the template correctly as to strand specificity (Reeder & Roeder, 1972; Yamamoto & Seifart, 1977; Orkin, 1978; Yang et al., 1980) and elongation and termination (Manley et al., 1979a). Recently, correct initiation of transcription in isolated nuclei has been demonstrated for both RNA polymerase II (Manley et al., 1979b) and RNA polymerase III (Marzluff et al., 1974; Vennstrom et al., 1978).

Accurate transcription, in terms of the correct use of promoters, has also recently been obtained by using appropriate cellular extracts with exogenous DNA templates (Wu, 1978; Ng et al., 1979; Weil et al., 1979). However, in these studies, transcription is observed to be promiscuous, all promoters being used, in contrast to the normal in vivo situation (Manley et al., 1980). In several studies, nuclear systems have been observed to maintain patterns of regulated (restricted) transcription [e.g., see Nguyen-Huu et al. (1978), Chan et al. (1978), Panyim et al. (1978), Bellard et al. (1977), and Yang et al. (1980)]. We wished to establish such a system using Drosophila as part of a study of the role of chromatin structure in determining the transcriptional state of genes.

The RNA polymerase activities of *Drosophila melanogaster* have been examined in some detail (Phillips & Forrest, 1973; Greenleaf & Bautz, 1975; Gross & Beer, 1975; Greenleaf et al., 1976). RNA polymerase I and II, the most closely studied species, exhibit salt optima and α-amanitin sensitivities typical of other eukaryotic polymerases (Greenleaf et al., 1976). A presumptive RNA polymerase III has been observed in partially purified preparations (Phillips & Sumner-Smith, 1977). Previous reports have also demonstrated transcriptional activities in nuclei isolated from various tissues of *Drosophila melanogaster* (Stein, 1976; Gross & Ringler, 1979; Sumner-Smith & Phillips, 1979).

We report here a study of the products of RNA synthesis in nuclei isolated from *Drosophila melanogaster* embryos. We were aided in this work by the availability of recombinant DNA plasmids, which have been utilized in an analysis of the selectivity and fidelity of the in vitro transcription reaction. The plasmid 12D1, which contains a segment of the *Drosophila* 5S gene region (Artavanis-Tsakonas et al., 1977), has been used to show that 5S RNA, an RNA polymerase III product, is initiated and elongated correctly in these nuclei. We have also examined transcription from several of the heat shock loci. [For a review of the heat shock response, see Ashburner & Bonner (1979)]. The transcripts from the *hsp*70 gene regions were analyzed by hybridization to the plasmids pPW 223 from 87A and pPW 232s and pPW 248 from 87C1 (Livak et al., 1978; Holmgren et al., 1979). For this particular experiment, RNA was synthesized in vitro in the presence of 5′-mercuri-UTP to allow for its selective isolation from preexisting RNA by thiol-affinity chromatography, greatly simplifying the hybridization analysis (Dale & Ward, 1975; Smith & Huang, 1976). This study has shown that these sequences are faithfully transcribed in vitro by RNA polymerase II in nuclei from heat-shocked, but not control, embryos. Additionally, the plasmid 15.1, which contains the 5′ end of the *hsp*68 gene from locus 95D (Holmgren et al., 1979; R.

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Holmgren, R. Morimoto, R. Blackman, and M. Meselson, unpublished experiments), has been used to examine the accuracy of transcription in this system; the results obtained are consistent with the occurrence of accurate initiation. The results indicate that gene activation by heat shock results in an alteration of the template at specific loci to an active state that is stable during nuclear isolation and independent of high temperature or special ionic conditions.

Materials and Methods

Embryos. Embryos (6–18 h) of *Drosophila melanogaster* Oregon R were grown and harvested as previously described (Elgin & Miller, 1978). After collection, embryos to be used in routine analysis were frozen at −80 °C. For studies of the heat shock response, half of the collection was immediately frozen in liquid nitrogen as a control sample, while the other half was incubated at 35 °C in *Drosophila* Ringer's solution (7 g of NaCl, 0.35 g of KCl, and 0.21 g of $CaCl_2$ per L) (30 g of embryos per 500 mL of Ringer's) for 20 min with vigorous shaking. The embryos were subsequently collected on a Nitex filter and immediately frozen in liquid nitrogen. Such samples could be stored at −80 °C for several months without deleterious consequences.

Nuclei. Nuclei were prepared by a procedure derived from that of Phillips & Forrest (1973). Approximately 30 g of partially thawed embryos was dechorionated in 300 mL of a solution of 50% bleach, 1% Triton X-100, and 1% NaCl by stirring for 2–5 min. The embryos were collected on a Nitrex screen and washed well with cold tap water, and the mass was pressed dry in the Nitrex using paper towels. All subsequent steps were performed at 0–4 °C. The eggs were homogenized in three batches with ten hand strokes of a type C Teflon–glass homogenizer (Thomas Scientific Apparatus) in ca. 30 mL of buffer A [1.0 M sucrose, 4 mM $MgCl_2$, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA), 2 mM β-mercaptoethanol, and 10 mM sodium *N*-2-(hydroxyethyl)piperazine-*N*'-3-propanesulfonate (NaHepps) (Calbiochem), pH 7.5]. The homogenates were filtered through two layers of Miracloth (Chicopee Mills) and collected. The retentate of this step was rehomogenized in one batch with buffer A and again filtered through two layers of Miracloth. The total volume of filtrate was taken up to 175 mL with buffer A and was then spun at 400*g* for 10 min in a fixed-angle rotor. The pellet of debris was discarded, and the supernatant was further spun at 4400*g* for 10 min to pellet the nuclei. The nuclei were resuspended by vortexing in buffer A, total volume of 25 mL, layered over 2 volumes of buffer B (1.75 M sucrose, 2 mM $MgCl_2$, 0.1 mM EGTA, 2 mM β-mercaptoethanol, and 10 mM NaHepps, pH 7.95) and spun at 25000*g* for 30 min in a swinging bucket rotor. The supernatant was aspirated off, and the nuclei were resuspended with the aid of a glass–Teflon homogenizer in a minimal volume (typically 1 mL) of buffer C (1.35 M sucrose, 2 mM $MgCl_2$, 0.1 mM EGTA, 2 mM β-mercaptoethanol, and 10 mM NaHepps, pH 7.95). The yield is usually (5–10) × 10^9 nuclei, ca. 10–20% of the estimated embryonic nuclei.

Transcription Reaction. Unless otherwise noted, all transcription reactions were carried out at 22 °C under the following conditions: 5 mM $MgCl_2$, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 75 mM KCl, 25 mM $(NH_4)_2SO_4$, 270 mM sucrose, 50 mM NaHepps, pH 7.95, and 0.25 mM of each rXTP. In addition, reaction mixtures for time course experiments contained bovine serum albumin (BSA) at 1 mg/mL; those reactions from which the RNA was to be run on gels or fingerprinted contained rat liver RNase inhibitor prepared through the DEAE-Sephadex A-50 step of the procedure of Gribnau et al. (1969). Where used, 5'-mercuri-UTP (Boehringer) replaced UTP at 0.25 mM. Labeled ribonucleotides (Amersham or New England Nuclear, approximately 350 Ci/nmol) were used as indicated in the figure legends. Nuclei were typically at (4–10) × 10^8/mL in the reaction mixture.

For time course determinations, 50-μL aliquots of the reaction mixture were removed at appropriate times and spotted onto 25-mm DE-81 (DEAE-cellulose, Whatman) paper circles previously moistened with 0.1% CETAB (hexadecyltriethylammonium bromide) and 0.1 M EDTA. The filters were washed 3 times at 10 mL per filter with 0.3 M ammonium formate, pH 8.0, and 3 times at 10 mL per filter with 0.25 M ammonium carbonate, pH 8.0 (Fodor & Doty, 1977). After rinsing with ethanol and air drying, they were counted in Scintiverse (Fisher).

Isolation of RNA. Typically, 250 μL of the reaction mixture was added, after 30 min of transcription at 22 °C, to 5 mL of lysis solution [2% sodium dodecyl sulfate ($NaDodSO_4$), 7 M urea, 0.35 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0], and the nucleic acids were extracted twice at 20–25 °C with phenol solution (100 mL of phenol, 100 mL of chloroform, 2 mL of isoamyl alcohol, and 0.2 g of 8-hydroxyquinoline) (Holmes & Bonner, 1973). After precipitation with 2 volumes of ethanol, the material was treated with 10 μg of DNase (Sigma) in 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM Tris-HCl, pH 8.0, for 30 min at 37 °C. The DNase had been made RNase free by a combined procedure of iodoacetate treatment (Zimmerman & Sandeen, 1966) followed by gel filtration (Holmes & Bonner, 1973). The RNA was finally either ethanol precipitated from high salt (2.5 M ammonium acetate) or passed over a Sephadex G-50 (Pharmacia) column in TNE (5 mM NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl, pH 7.8) and ethanol precipitated from this buffer made 2% in sodium acetate.

The following protocol was used to fractionate RNA polymerized in the presence of HgUTP. The isolated RNA was heated at 95 °C for 10 min in TNE and then placed in an ice bath. Sodium acetate was added to 0.2 M. The RNA was applied to a thiopropyl-Sepharose 6B (Pharmacia) column which had first been washed with HSB (0.2 M sodium acetate, 0.1 mM EDTA, and 10 mM Tris–acetate, pH 7.8) plus 0.2 M dithiothreitol, and then with HSB. After application of the RNA, the column was further washed with HSB. The bound RNA was eluted with HSB plus 0.2 M mercaptoethanol and maintained in this solution for 30 min at room temperature to promote demercuration. The RNA was then ethanol precipitated with 100 μg of yeast carrier RNA.

Drosophila tissue culture cells (Schneider's line 2) were grown overnight in complete media with 1 mCi of $^{32}P_i$ per 2 mL of cells to obtain labeled RNA synthesized in vivo. Total RNA was purified as described above.

Plasmids. Clones containing the tet plasmids pPW 223, pPW 232s, and pPW 248 were obtained by Dr. Ken Livak (Livak et al., 1978) in a screen of *Drosophila melanogaster* Oregon R library (Gergen et al., 1979). Plasmid pPW 223 is from the 87A region and contains the *hsp*70 message sequence and the adjacent 3' nontranscribed region. Plasmids pPW 232s and pPW 248 are from the 87C1 region and contain the *hsp*70 message sequence as well as transcribed but nontranslated sequences. The tet plasmid 15.5, a subclone of the phage clone λ15 (R. Holmgren, R. Morimoto, R. Blackman, and M. Meselson, unpublished experiments; Maniatis et al., 1978), was constructed and made available to us by Dr. Robert Holmgren. It contains the start site of transcription for the

*hsp*68 gene on a *Pst*I fragment which has been inserted into the *Pst*I site of pBR 322. The amp plasmid 12D1 contains multiple copies of the 5S RNA genes of *Drosophila melanogaster* (Artavanis-Tsakonas et al., 1977).

Bacteria (*Escherichia coli* HB101) were grown in media containing either tetracycline at 15 μg/mL or ampicillin at 200 μg/mL, as appropriate. All were amplified with chloramphenicol at 200 μg/mL.

Plasmids were prepared by using the cleared lysate procedure of Godson & Sinsheimer (1967). The lysate was added to an equal volume of lysis solution, phenol extracted as described above, and ethanol precipitated. The pellet of nucleic acids was dissolved in TNE and digested with pancreatic RNase at 100 μg per 300 mL of original culture volume for 30 min at 37 °C. The material was then passed over a Sephadex G-75 (Pharmacia) column in TNE and used directly. Restriction enzymes (New England Biolabs) were used under the conditions specified by the supplier.

Gel Electrophoresis. RNA was analyzed on agarose (Seakem) or acrylamide (Bio-Rad) gels 15 cm long × 17 cm wide × 1.5 mm deep using an electrophoresis (E) buffer of 0.05 M H_2BO_3, 0.005 M $Na_2B_4O_7$, and 0.01 M Na_2SO_4 (Chandler et al., 1979). Gels contained methylmercuric hydroxide (CH_3HgOH) (Alfa Ventron, Danvers, MA) at a concentration of 10 mM in agarose gels and 20 mM in acrylamide gels. Electrophoresis was carried out at 30 mA for agarose gels and 50 mA for acrylamide gels with recirculation of buffer. The sample buffer was 0.1 × E buffer, 20 mM CH_3HgOH, and 10% glycerol. Labeled molecular weight standards were provided by using restriction fragments from well-characterized recombinant plasmids; the fragments were labeled by using the DNA polymerase Klenow fragment (Boehringer) to fill in with [α-^{32}P]dXTP's the 5′ overhang ends (Holmgren et al., 1979). The gels were dried on Whatman 3 MM paper prior to autoradiography.

DNA plasmid fragments from restriction digests were separated on horizontal 1% agarose gels using a TAE buffer (5 mM sodium acetate, 1 mM EDTA, and 40 mM Tris–acetate, pH 7.8).

Hybridization to and Elution from Filters. 5S RNA for further analysis was obtained via the following protocol. Restricted plasmid DNA was bound to 25-mm diameter BA85 nitrocellulose filters (Schleicher & Schuell) using the procedure of Melli et al. (1975). In general, 25 μg of the plasmid 12D1 was applied to each filter. The filters were prehybridized for 1–2 h at 37 °C with 50% formamide (Fluka), 0.75 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$, 100 μg/mL yeast RNA, and 50 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate) (NaPipes), pH 6.8, RNA (25 μg) synthesized in vivo or in vitro was added to each filter in 1.5 mL of the above buffer, and hybridization was allowed to proceed for 18 h at 37 °C. The filters were washed 2 times at 37 °C with the buffer minus the yeast RNA. Bound RNA was eluted in a single step with 1 mL of 90% formamide, 1 mM EDTA, 0.1% $NaDodSO_4$, and 50 mM NaPipes, pH 6.8, at 65 °C for 10 min. The eluted material was precipitated with 2 volumes of ethanol using 25 μg of yeast RNA as carrier. The pelleted RNA was analyzed directly on gels.

5S RNA to be analyzed by fingerprinting was isolated by hybridization to nitrocellulose filters prepared as above, using the prehybridization, hybridization, and washing procedures of Wahl et al. (1979) which decreases background hybridization. Elution from the filters was as described above.

Analysis of in Vitro Heat Shock Gene Transcripts. The mercurated transcripts were used in a general analysis of transcription as follows. DNA restriction fragments were separated on agarose gels as described above. The gels were blotted onto DBM paper (Alwine et al., 1979) and prehybridized following the protocol of Wahl et al. (1979). After demercuration and ethanol precipitation, the in vitro transcripts were hybridized to the filters. Hybridization and washing of the filters were carried out according to the procedure of Wahl et al. (1979) except that NaPipes (pH 6.8) was substituted for phosphate buffer and yeast RNA was substituted for denatured salmon sperm DNA. Hybridization was allowed to proceed for 18 h.

The following protocol was employed to analyze transcription at the 5′ end of a heat shock gene in vitro. A 25-μg sample of total RNA isolated from heat-shocked embryo nuclei after in vitro transcription was hybridized to a gel-purified *Hin*fI fragment of the plasmid 15.1 containing the start site of transcription (Holmgren et al., 1979). The reaction was stopped by adding 400 μL of 2 mM EDTA and 0.2 M Tris-HCl, pH 7.5, and 4 μg of RNase T_1 (P-L Biochemicals) to 100 μL of the hybridization mixture and incubating the solution at 37 °C for 30 min. After passage over a Sephadex G-50 column in TNE, the material was treated batchwise with oligo(dT) resin and then ethanol precipitated with 25 μg of carrier RNA. The standard of material synthesized in vivo was provided by RNA from Schneider's cells incubated with $^{32}P_i$ at 25 °C for 4 h and subsequently at 35 °C for 1 h. Total cellular RNA was isolated, and the appropriate transcript was subsequently isolated by using the above protocol.

The precipitates were dissolved in an appropriate buffer and heated at 95 °C for 10 min before subsequent enzymatic analyses.

Fingerprinting. RNA (12.5 μg) was digested with 1–2 μg of RNase T_1 in 10 μL of 2 mM EDTA and 10 mM Tris, pH 7.5, for 1 h at 37 °C. The material was spotted at the origin (2 cm from either edge of a corner) of a 20 × 20 cm poly(ethyleneimine)–cellulose plate (Cel MN 300 PEI, Brinkmann), and the plates were developed by using the two-dimensional system of Mirzabekov & Griffin (1972). Briefly, in the first dimension, development is by ascending chromatography at room temperature with 1.4 M lithium formate and 7 M urea, pH 3.4, to 10 cm above the origin. The plates are then transferred without drying to 1.8 M lithium formate and 7 M urea, pH 3.4, for continued development to 17 cm above the origin. After being washed with methanol and air-dried, the plates are developed in the second dimension by ascending chromatography at room temperature with 0.8 M LiCl, 7 M urea, and 20 mM Tris-HCl, pH 8.0, to 17 cm above the origin. The plates are then methanol washed, dried, and exposed to X-ray films.

RNase P1. RNA (5–10 μg) in 20 mM sodium acetate, pH 5.3, was digested with 1 unit of RNase P1 (Boehringer-Mannheim) for 15 min at 37 °C. The material was directly analyzed by ascending chromatography on PEI plates developed with 0.75 M KH_2PO_4, pH 3.4 (Cashel et al., 1969).

Autoradiography. All autoradiographs were produced on Kodak X-OMAT type R film using a Du Pont Cronex lightning plus intensifying screen according to the procedure of Laskey & Mills (1977).

Results and Discussion

Some General Aspects of the Reaction. As part of our initial observations in this system, we examined the effects of varying divalent cation concentrations on several general aspects of the reaction. Presented in Figure 1 are time courses of the reaction from experiments done under differing ion concentrations chosen on the basis of results reported by others.

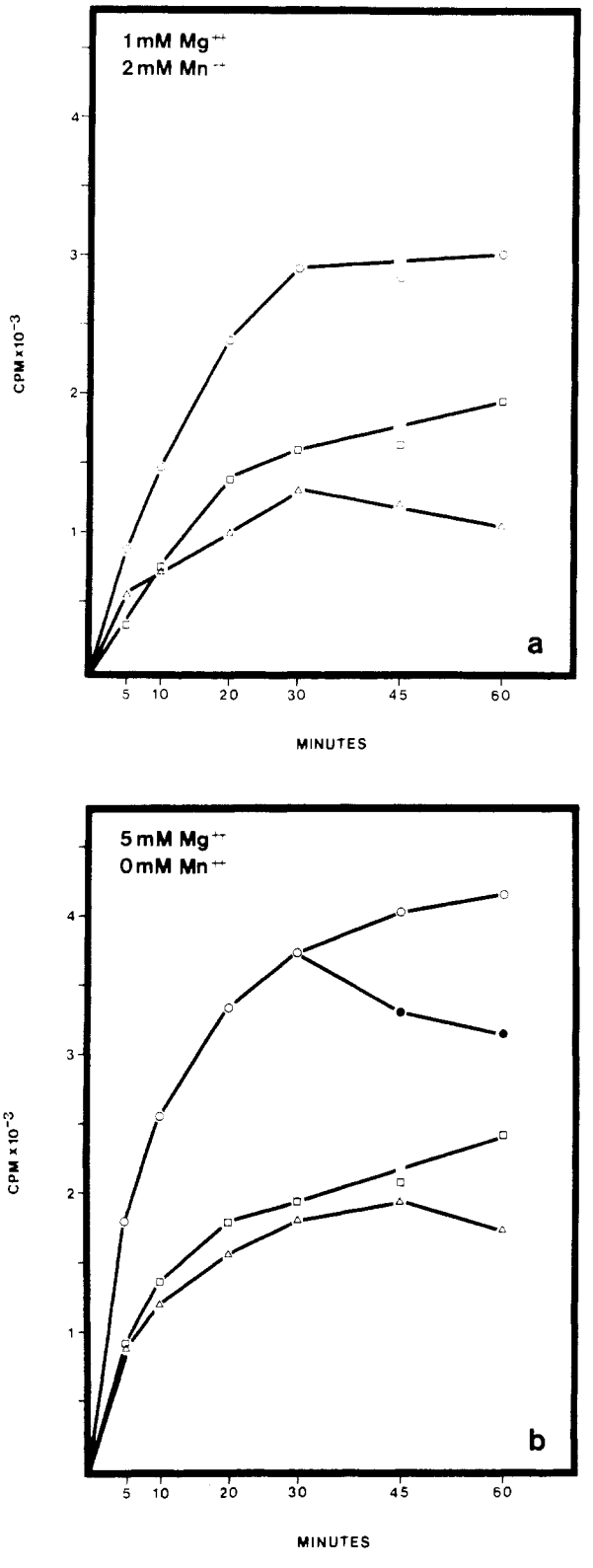

FIGURE 1: Reaction time courses under differing divalent cation concentrations. The standard reaction mixture for divalent cations has been either (a) changed to 1 mM $MgCl_2$ and 2 mM $MnCl_2$ or (b) unchanged. (○) Total label counts incorporated; (□) α-amanitin-insensitive activity, that is, label incorporated when 20 μg/mL α-amanitin is added to the standard reaction mixture; (△) α-amanitin-sensitive activity, determined as the difference of (○) and (□); (●) actinomycin D added to 100 μg/mL at 30 min time point. In this particular experiment; nuclei were at 4 × 10^8/mL (145 μg of DNA/mL). Label was [α-^{32}P]CTP at 64 mCi/mmol (4 μCi per 250 μL of reaction mixture). Maximal counts incorporated represent 3.86 pmol of CMP/μg of DNA or approximately 0.5 μg of RNA/100 μg of DNA.

They represent in one case (Figure 1a) those conditions (1 mM $MgCl_2$ and 2 mM $MnCl_2$) which provide an averaged optimum for the combined *Drosophila* RNA polymerase activities (Gross & Beer, 1975) and in the other case (Figure 1b) those conditions (5 mM $MgCl_2$ and 0 mM $MnCl_2$) which have been reported to be important for correct initiation and elongation by polymerase III (Birkenmeier et al., 1978). The latter condition, as will be shown, also maximizes production of several discrete species of RNA in this system. In both situations, total counts incorporated increase with time through the length of the experiment, although the rate continues to decrease. The suprising result is that the former conditions give the lesser degree of total label incorporation. In addition, under these conditions, the reaction slows dramatically after 30 min.

The inhibitor α-amanitin allows the separation of this reaction into two components: the sensitive polymerase II activity (Phillips & Forest, 1973; Gross & Beer, 1975; Greenleaf et al., 1976) and the resistant, and therefore combined, polymerase I and III activities (Greenleaf et al., 1976; and discussion below). The rate decrease characteristic of this system can, with the use of α-amanitin, be seen to be a combination of at least two types of kinetics. In the case of polymerases I and III, after an initial period of rapid synthesis the rate of synthesis decreases but then remains linear for the entire period of the experiment. The polymerase II reaction is characterized by a period of polymerization followed by the onset of apparent net degradation of polymerized material; this degradation may be largely responsible for the observed overall reaction rate decrease. The effect is most pronouced in the presence of Mn^{2+} (Figure 1a). A general detrimental effect on Mn^{2+} on nuclear transcription has been noted by others, particularly in the presence of Mg^{2+} (Yamamoto & Seifart, 1977; Panymin et al., 1978). Also shown in Figure 1b is the effect on the level of incorporated counts when sufficient actinomycin D is added to the nuclei to completely stop transcription; as there is no RNA being produced, the observed decrease again demonstrates the endogenous degradation of material. RNA degradation has been observed in other *Drosophila* nuclear transcription systems (Gross & Ringler, 1979; Sumner-Smith & Phillips, 1979).

Under both reaction conditions shown, polymerase II activity represents a maximum of about 50% of the total nucleotide incorporation. This activity is inhibitable by as little as 1 μg/mL α-amanitin, but higher amounts (e.g., 20 μg/mL) have been routinely used to ensure total inhibition. It is noteworthy that amounts as high as 100 μg/mL caused no further inhibition of the combined polymerases I and III transcriptional activity when assayed by incorporation of labeled ribonucleotides. This is well within the range of inhibition of polymerase III activity in other eukaryotic organisms (Weimann & Roeder, 1974; Birkenmeier et al., 1978), and levels this high have been reported to inhibit an additional activity referred to as polymerase III in isolated *Drosophila* nuclei (Phillips and Sumner-Smith, 1977). However, when polymerase III activity was assayed directly by monitoring 5S RNA production, α-amanitin at 100 μg/mL caused no decrease in synthesis over the control level (Miller, 1980). This is consistent with the reported resistance of another insect RNA polymerase III, that of *Bombyx mori* (Sklar et al., 1976).

The total reaction products synthesized with the divalent cation concentrations of Figure 1 as well as one additional set of frequently used conditions [5 mM $MgCl_2$ and 1 mM $MnCl_2$ (Reeder & Roeder, 1972; Marzluff et al., 1974; Ernest et al.,

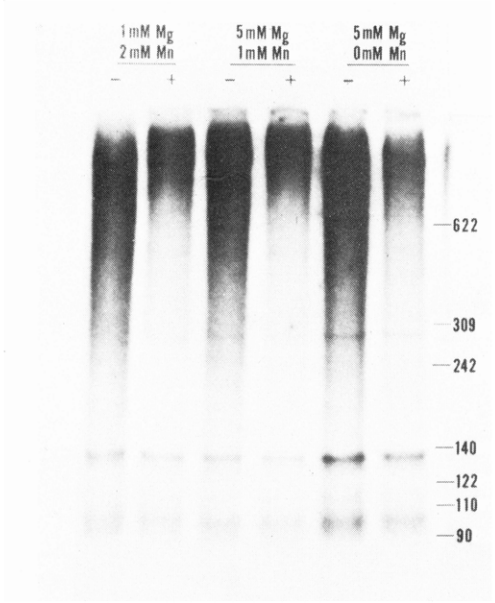

FIGURE 2: Products of the transcriptional reaction with differing divalent cation concentrations. RNA was isolated from nuclei after incubation in the standard reaction mixture with the exception that the divalent cation concentrations were changed as indicated in the figure. Shown is an autoradiogram of the RNA after electrophoresis on a 4–12% acrylamide gradient gel containing CH_3HgOH. In each case, the effect of α-amanitin at 20 μg/mL (+) is compared to the control without α-amanitin (–). Label was [α-^{32}P]CTP. Each lane received 5 μg of total nuclear RNA. The sizes, in nucleotides, are from an *Msp*I digest of pBR 322.

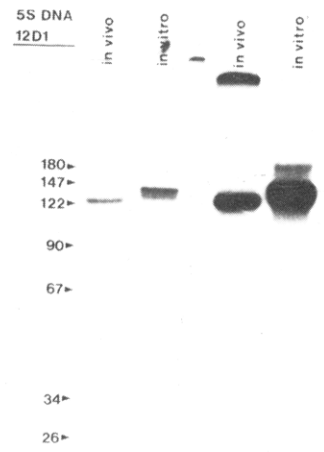

FIGURE 3: Hybridization-purified RNA complementary to 5S DNA. In vivo ($^{32}P_i$ labeled) and in vitro ([α-^{32}P]CTP labeled) synthesized RNA was purified by hybridization to and elution from nitrocellulose filters on which was bound the *Drosophila* 5S DNA plasmid 12D1. Shown is an autoradiogram of a 10% acrylamide–CH_3HgOH gel in which the purified material has been electrophoresed. The left lanes are an 8-h exposure, and the right lanes are a 5-day exposure of the same gel. The size markers are provided by an *Msp*I digest of pBR 322.

1976)] have been analyzed by gel electrophoresis and are shown in Figure 2. The increased incorporation of labeled nucleotides seen in the previous experiment is reflected in an increase in the production of several RNA species. Although an increase in α-amanitin-sensitive activity can be detected, those RNAs most obviously increased in the absence of Mn^{2+} (those at 100, 135, and 300 nucleotides) are produced by α-amanitin-resistant activities. The 135-nucleotide species is the 5S RNA precursor, an RNA polymerase III product (see below). This negative effect on Mn^{2+} on RNA polymerase III transcription of the 5S genes has also been observed in other systems (Yamamoto & Seifart, 1977; Birkenmeier et al., 1978). With *Xenopus* oocyte nuclear extracts, where Mn^{2+} was used in the absence of Mg^{2+}, this was attributed to an increase in aberrant transcription (Birkenmeier et al., 1978). As a result of these studies, we have chosen not to use Mn^{2+} in our standard reaction mixture.

Transcription of the 5S DNA in Isolated Nuclei. The 5S RNA of *Drosophila* is well-characterized and as such is a logical first choice of product to use in an analysis of the specificity of an in vitro nuclear transcription system. The 5S RNA has been shown to be produced as a 135-nucleotide precursor which is processed to the 120-nucleotide mature species by removal of 15 nucleotides from the 3′ end (Rubin & Hogness, 1975; Jacq et al., 1977). Both RNAs have been sequenced, and the 5′ end has been shown to have the structure pppGp... (Rubin & Hogness, 1975; Benhamou & Jordan, 1976; Jacq et al., 1977).

The nature of the in vitro transcription product homologous to 5S DNA was analyzed by hybridizing total in vitro made RNA to filters containing the 5S DNA recombinant plasmid 12D1 (Artavanis-Tsakonas et al., 1977), eluting the hybridized material, and comparing this by several techniques to a similarly purified standard synthesized in vivo. Presented in Figure 3 are the results of comparative gel electrophoresis. As expected, in vivo synthesized 5S RNA consists of a band of approximately 120 nucleotides. This band has a correlate in the in vitro synthesized RNA sample, but in this case, the predominant species is one migrating as would be expected for the 5S RNA precursor. Synthesis in vitro in these nuclei is thus producing an RNA species of a size consistent with correct elongation and termination of the 5S RNA transcript. We have not established a precursor–product relationship for the two species synthesized in vitro, so it remains possible that the two different sized molecules are the products resulting from two different chain termination events. It seems probable, however, that the 135-nucleotide species is being processed in these nuclei to the mature 120-nucleotide presumptive 5S RNA. It has been shown that in vivo the precursor is short-lived (Jacq et al., 1977). The preponderance of precursor over product in vitro would indicate that the processing machinery, while intact, is not functioning at in vivo rates.

Whether or not initiation of transcription is occurring for a particular RNA species in a given system is of course of great interest. We have approached this question for the 5S RNA genes in two ways. First, we have looked for homology between RNase T_1 fingerprints of in vivo and in vitro synthesized material. Homology would firmly establish that the in vitro synthesized RNA was in fact 5S RNA and would furthermore be indicative of an in vitro initiation event. Second, we have examined the material synthesized in vitro for the presence of a labeled initiating nucleotide as direct proof of an initiation event.

Presented in Figure 4 are comparative RNase T_1 fingerprints of hybridization-purified 5S RNA synthesized in vivo with $^{32}P_i$ as label and in vitro with [α-^{32}P]GTP as label. As can be seen, the fingerprints of the RNAs are nearly identical. Although the base composition of the various oligonucleotides has not been determined for this particular thin-layer chromatography system, the differences observed can be accounted for in terms of what is known about the structure of the 5S RNA and its precursor. In the in vivo synthesized RNA, the terminal oligonucleotide CCU_{OH} should be observed, but it

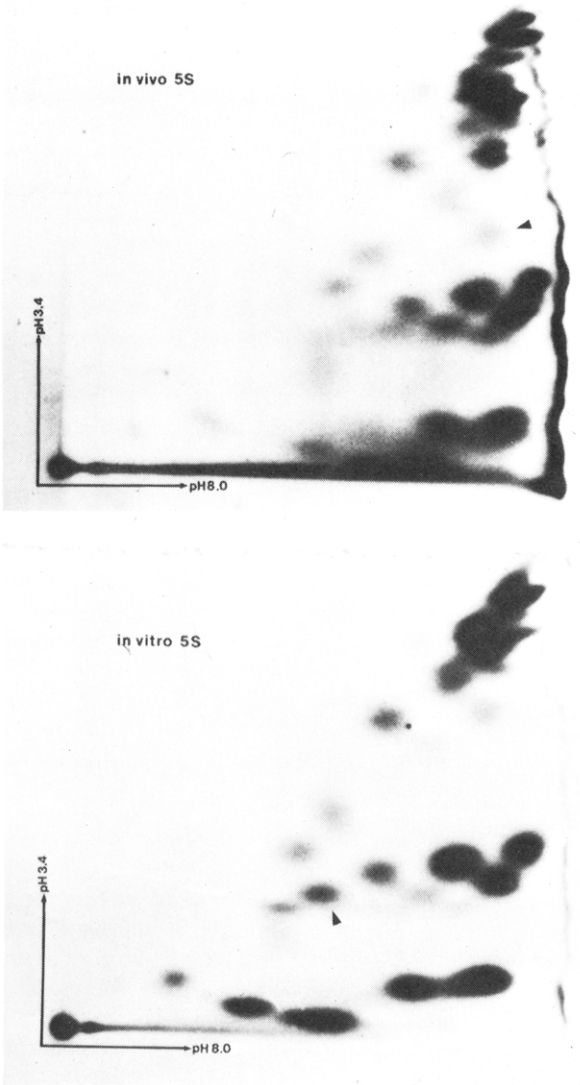

FIGURE 4: Comparative RNase T_1 fingerprints of hybridization-purified RNA complementary to 5S DNA. In vivo ($^{32}P_i$ labeled) and in vitro ([α-^{32}P]GTP labeled) synthesized RNA was purified by hybridization to and elution from nitrocellulose filters on which were bound 12D1 DNA. The RNAs were treated with RNase T_1, and the digests were separated by two-dimensional chromatography on PEI–cellulose plates. Autoradiograms of these plates are shown. The arrows locate spots discussed in the test.

will not be labeled in the in vitro synthesized RNA given the protocol of the experiment. The presence of a single additional G residue in the 15 nucleotides that are part of the precursor molecule, the predominant species of 5S RNA synthesized in vitro, leads to the observation of an additional oligonucleotide, CCUCGP, in that case. The likely candidates for these spots are indicated in Figure 4. The presence of all expected oligonucleotides in the fingerprint of the in vitro synthesized material indicates that RNA polymerase III is transcribing the entire length of the gene, a result consistent with correct initiation, elongation, and termination.

Direct evidence for initiation of transcription of the 5S RNA gene in vitro is provided by the recovery of a labeled pppG, the initiating nucleotide, from the 5′ end of the in vitro synthesized material. In this experiment, hybridization-purified in vivo or in vitro synthesized 5S RNA, labeled as in the previous experiments, was digested with RNase P_1, releasing 5′-nucleotide monophosphates from within the RNA chain. Also released is the nucleotide from the 5′ end, which for 5S RNA is predicted to be GTP. Because of the labeling regimen,

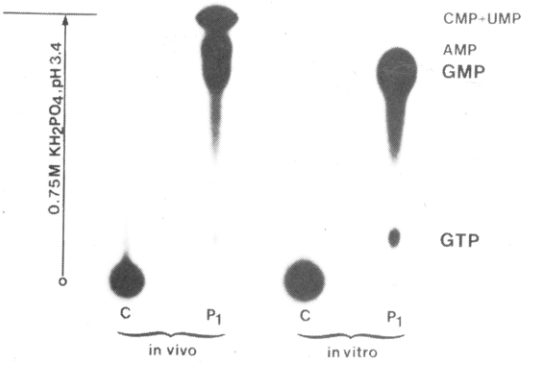

FIGURE 5: Identification of a labeled pppG at the 5′ end of 5S RNA. In vivo ($^{32}P_i$ labeled) and in vitro ([α-^{32}P]GTP labeled) synthesized, hybridization-purified 5S RNA was either left untreated (C) or digested with RNase P_1 (P_1). The material was chromatographed on a PEI–cellulose plate; an autoradiogram of this plate is shown. The location of standards is indicated.

one anticipates that in the in vivo synthesized RNA only GMP and, given initiation, GTP will be labeled. The results presented in Figure 5 demonstrate that this is exactly what is found; a labeled GTP is recovered in both the in vivo and in vitro synthesized 5S RNAs, establishing the in vitro initiation reaction.

The radioactivity present in the separated GTP and GMP nucleotides of the in vitro synthesized sample was determined. If it is assumed that every GTP recovered represents a 5S RNA chain initiated and fully elongated, it can be calculated that 6 times more RNA is initiated and made in vitro in 30 min than is produced from chains already initiated at the moment of isolation of the nuclei. The above assumption could lead to an overestimate of the contribution of the in vitro initiated material to the total pool of in vitro synthesized 5S RNA. If it were incorrect, however, one would expect that hybridization purification of the in vitro synthesized RNA would yield not only full-length transcripts but also material of a shorter length. An examination of Figure 3 shows that very little such material is observed. We therefore conclude that 5S RNA is both initiated, elongated, and terminated correctly in these nuclei; up to 80% of the synthesized material is estimated to result from in vitro initiation. This result is comparable with that obtained in other systems (Marzluff et al., 1974; Gilboa et al., 1977; Yamamoto & Seifart, 1977).

Transcription from Heat Shock Gene Loci in Isolated Nuclei. The heat shock response of *Drosophila* provides a dual means of assaying the fidelity of the in vitro transcription reaction. Because of the existence of recombinant plasmids containing the DNA from heat shock loci in which the domains of transcription have been well mapped (Livak et al., 1978; Holmgren et al., 1979), one may readily assay the accuracy of the in vitro transcription. In addition, the inducible nature of the heat shock response [reviewed by Ashburner & Bonner (1979)] makes it an ideal system in which to examine the ability of the isolated nuclei to maintain a correct representation of the in vivo state.

Nuclei from heat-shocked and control embryos were allowed to transcribe under the usual in vitro conditions for 20 min, and the in vitro synthesized RNA was isolated. The transcripts were assayed by hybridization to various recombinant plasmids

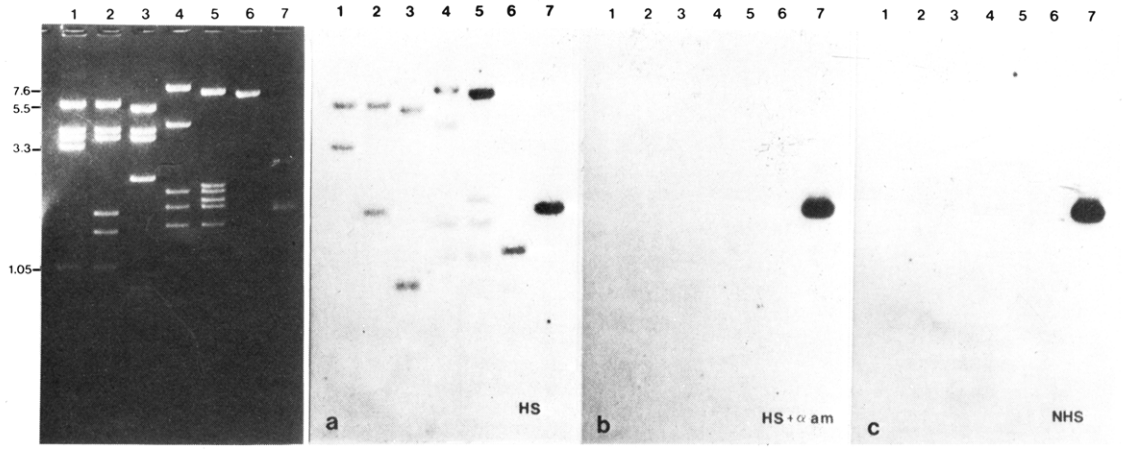

FIGURE 6: Heat shock gene transcription. Plasmids containing *hsp*70 gene segments were restricted and separated by electrophoresis on three identical agarose gels, and the gels were blotted onto DMB paper. The blots were hybridized with newly synthesized RNA ([α-^{32}P]CTP labeled, HgUTP substituting for UTP) isolated by using sulfhydryl-Sepharose chromatography. (a) Hybridized with RNA synthesized in nuclei isolated from heat-shocked embryos; (b) same as in (a), but α-amanitin was added to the reaction mixture at 20 μg/mL; (c) hybridized with RNA synthesized in nuclei isolated from the control embryos. Shown on the left is a representative ethidium bromide stained gel before blotting. Lane 1, *Bam*HI digest of pPW 223; lane 2, *Bam*HI, *Bgl*II digest of pPW 223; lane 3, *Bam*HI, *Sal*HI, digest of pPW 223; lane 4, *Hin*dIII digest of pPW 232s; lane 5, *Hin*dIII, *Xho*I digest of pPW 232s; lane 6, *Hin*dIII, *Pst*I digest of pPW 248; lane 7, *Hae*III digest of 12D1. This last sample was loaded when the other samples had migrated about one-half of the final distance. The sizes on the left are in kilobases.

containing segments of the Drosophila *hsp*70 heat shock genes. The plasmids had been appropriately restricted, the fragments separated by gel electrophoresis, and the gel blotted to DBM paper (Wahl et al., 1979). An essential aspect of this analysis is the use of the UTP analogue, 5′-mercuri-UTP. Because of its reactive nature toward sulfhydryl groups, newly synthesized RNA in which 5′-mercuriuridine is a constituent can be selectively isolated from the RNA which was preexisting in the nucleus using a sulfhydryl-Sepharose affinity column (Dale of Ward, 1975; Smith & Huang, 1976).

Presented in Figure 6a are the results of this type of analysis for transcription products of nuclei prepared from heat-shocked embryos. These are to be compared to the products from nuclei of the same preparation incubated in the presence of α-amanitin (Figure 6b), and to the products of nuclei prepared from control embryos (Figure 6c). Each filter also contains a digest of the 5S DNA clone, 12D1, as an internal control. Transcription of the 5S genes is, as shown above, insensitive to α-amanitin at the levels used here. It is also unaffected by heat shock (Rubin & Hogness, 1975; Spradling et al., 1975; Jacq et al., 1977). As can be seen, transcription in vitro mimics that in vivo is that there is no detectable transcription at the heat shock loci in nuclei from control embryos but readily detectable transcription at these loci in nuclei from heat-shocked embryos (cf. Figure 6a, c). This indicates that the nuclei maintain the in vivo state of gene expression during the course of isolation and in vitro transcription. It should be noted that as measured by incorporation of labeled ribonucleoside triphosphate, overall transcriptional activity is reduced by a factor of three in isolated heat-shocked nuclei when compared to the control preparation (Miller, 1980). Although the percentage of the newly synthesized RNA represented by heat shock gene transcripts has not been directly determined, we estimate from density scans of the labeled bands compared to the background that we are detecting at least a 25-fold increase of transcriptional activity at these loci.

A map of the plasmids used, indicating the regions of transcription in vivo (Livak et al., 1978), is presented in Figure 7. The restriction fragments to which newly synthesized RNA hybridized (i.e., those regions transcribed in vitro) are also indicated. Excellent homology between in vivo and in vitro patterns of transcription is observed. Both the 5′ and 3′

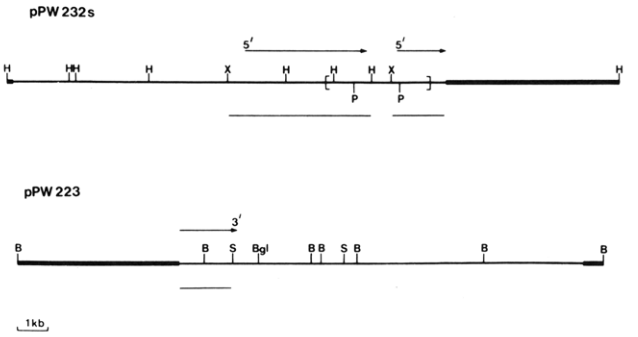

FIGURE 7: Map of the heat shock gene plasmids. Linearized maps of the plasmids pPW 223, pPW 232s, and pPW 248 are shown, redrawn from Livak et al. (1978). The vector, pMB9, is indicated in bold lines. The *Drosophila* DNA segment of pPW 248 is delineated by the brackets on the pPW 232s map. The lines above each plasmid indicate those regions mapped as transcribed in vivo with the direction of transcription (Livak et al., 1978). The lines below each plasmid indicate the segments to which in vitro transcripts hybridized, using the data of Figure 6a. B = *Bam*HI; Bgl = *Bgl*II; *H* = *Hin*dIII; *P* = *Pst*I; *S* = *Sal*I; X = *Xho*I.

boundaries of the transcribed regions are correctly observed in vitro at this level of resolution; only those sequences known to be transcribed in vivo are transcribed in vitro. Those factors which limit RNA polymerase in vivo are still operating in these nuclei.

In another series of experiments, using the in situ hybridization of in vitro synthesized RNA to polytene chromosomes, we observed that, with the notable exception of the histone locus [see also Spradling et al. (1975)], the bulk of polymerase II transcription is abolished in nuclei isolated from heat-shocked embryos (D. W. Miller, R. Blackman, and S. C. R. Elgin, unpublished experiments). Therefore, both aspects of transcriptional control evident in cells during a heat shock response are maintained in these isolated nuclei: those genes which would otherwise be active are silent, and the heat shock genes are active.

Of additional interest is the fact that α-amanitin abolishes synthesis at all regions of transcription of loci 87A and 87C1, indicating that all transcripts are products of synthesis by RNA polymerase II (cf. Figure 6a,b). It has been shown that the bulk of transcription from the euchromatic arms of Dipteran

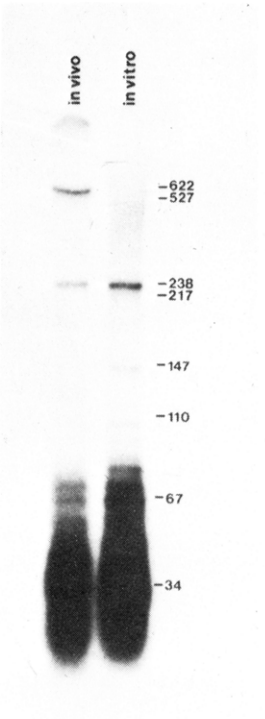

FIGURE 8: Comparative gel electrophoresis of heat shock RNA protected from nuclease digestion. Total RNA synthesized in vivo in heat-shocked Schneider's cells ($^{32}P_i$ label) or in vitro in nuclei isolated from heat-shocked embryos ([α-^{32}P]GTP label) was hybridized to a restriction fragment containing the 5′ end of the *hsp*68 gene. The solution was treated with RNase T_1 and the protected material electrophoresed on a 5% acrylamide–CH_3HgOH gel. An autoradiogram of the gel is shown. The size markers are in bases and are from an *Msp*I digest of pBR 322.

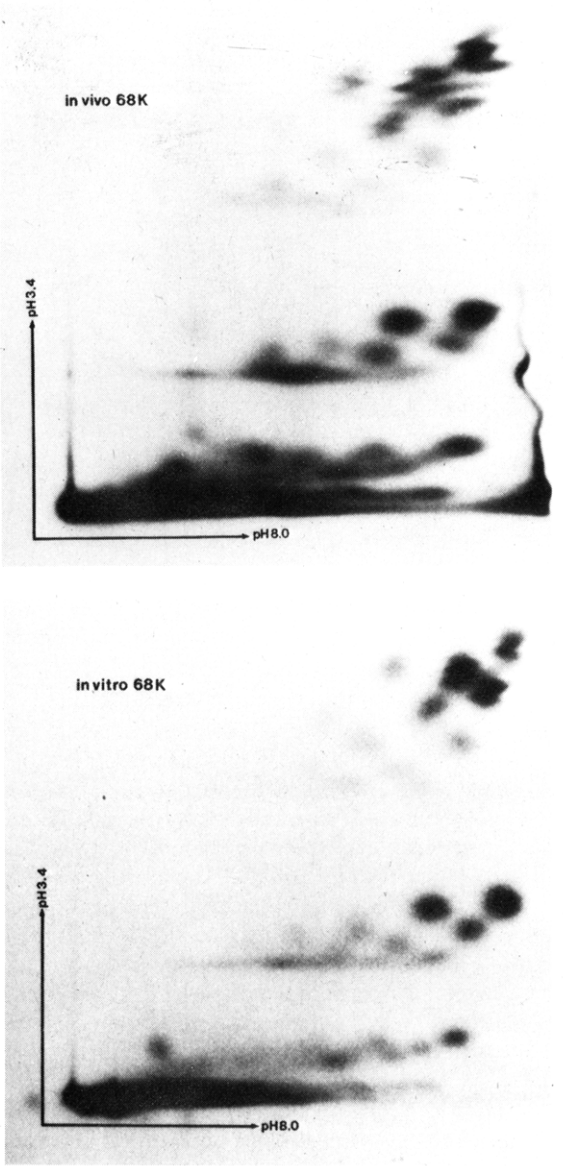

FIGURE 9: Comparative RNase T_1 fingerprints of heat shock RNA protected from nuclease digestion. The RNAs of Figure 8 were further treated with RNase T_1. The digests were separated by two-dimensional chromatography on PEI–cellulose plates. Autoradiograms of these plates are shown.

polytene chromosomes is sensitive to α-amanitin (Egyhazi et al., 1972; Stein, 1976; Hameister, 1977). Also, antibodies directed against RNA polymerase II react with the induced heat shock puffs of polytene chromosomes (Plagens et al., 1976; Elgin et al., 1977). Given these results and the role of this polymerase in transcribing hnRNA- and mRNA-like sequences, it is not suprising that RNA polymerase II is responsible for transcription of the *hsp*70 message sequence. It is perhaps more noteworthy that the regions adjacent to the *hsp*70 message sequence are transcribed by RNA polymerase II. These sequences are of middle repetitious frequency (Lis et al., 1978) and have no clearly demonstrated role in the heat shock response; no translation products have been identified (Livak et al., 1978). Sequences that might be considered analogous to these are present near the globin gene sequences in humans; however, these are transcribed (in vitro) by RNA polymerase III (Duncan et al., 1979).

The hybridization experiments indicate which regions are transcribed by RNA polymerase but are limited in resolution. It is of particular interest to investigate transcription at the 5′ end of the genes. For this series of experiments, a clone, 15.1, containing the 5′ end of the gene coding for *hsp*68, has been used (R. Holmgren, R. Morimoto, R. Blackman, and M. Meselson unpublished experiments). By experiments analogous to those described above, this transcript has been shown to be a polymerase II product (Miller, 1980). The transcripts produced in vivo and in vitro from the 5′ end of the *hsp*68 gene were compared by using the following protocol. A 335-base pair (bp) *Hin*fI fragment from plasmid 15.1, containing the start site of transcription plus approximately 230 nucleotides downstream, was hybridized with, in one case, RNA isolated from Schneider's cells heat shocked in the presence of $^{32}P_i$ and, in the other case, with RNA isolated from nuclei of heat-shocked embryos after their in vitro incubation in the presence of [α-^{32}P]GTP. The hybridization reaction mixtures were subsequently treated with RNase T_1. As shown in Figure 8, electrophoresis of the nondigested material in a denaturing gel reveals a sharp band at 230 nucleotides from both the in vivo and in vitro synthesized RNA as well as smaller bands, present and homologous, in both samples. These small bands are apparently the products of the nuclease nicking the hybridizing material and, in effect, constitute a partial RNase T_1 map of the RNA. The higher molecular weight band present in the in vivo synthesized material is of unknown origin. It is presumably a double-stranded RNA present in the tissue culture cells.

When the RNA–DNA hybrids are denatured and the RNA is fingerprinted by using RNase T_1, one sees a great similarity between the oligonucleotide patterns of the in vivo and in vitro produced RNA. The data are presented in Figure 9. There is, for the most part, a direct correspondence between the patterns. This would be expected only if the transcription in

vitro covered the entire length of this region, including the 5′ end. The data from these experiments indicate that in vitro one obtains a transcription by RNA polymerase II of the 5′ region of the *hsp*68 gene which is comparable to that occurring in vivo, and the data are consistent with the occurrence of correct initiation in vitro.

Evidence has been presented by others suggesting that the heat shock messenger RNAs, when analyzed as a group, are capped (Levis & Penman, 1978). We have examined an RNase P_1 digest of the 230-nucleotide-long protected RNA fragment from the 5′ end of the *hsp*68 gene. In neither the in vivo nor the in vitro synthesized material were we able to identify an alkaline phosphatase resistant structure as would be expected if this RNA were capped (Miller, 1980). It is possible that our method of preparation of the protected fragment resulted in a loss of the cap structure from the RNA. It is also possible that the RNA is not capped in this nuclear system. This question is currently unresolved.

Conclusions

The analysis presented here of in vitro transcription in nuclei isolated from *Drosophila melanogaster* embryos demonstrates that in several regards these nuclei maintain a faithful and accurate representation of the in vivo state. There is extensive initiation of transcription at the 5S locus, resulting in a 5S RNA transcription product comparable to the that of the in vivo material. Utilizing nuclei isolated from heat-shocked embryos, we have also shown that these nuclei maintain the specific, active state of the heat shock gene loci; the heat shock genes are transcribed in a manner consistent with the in vivo situation.

The stability of the induced state as demonstrated by these experiments has implications for models of heat shock gene control. Because the transcription reactions are carried out at 22 °C rather than at some higher temperature, temperature alone cannot be the required effector. Furthermore, neither can some unique set of ionic conditions be responsible, as these are specified by the reaction mixture. Rather, there must be some direct and stable alteration of the RNA polymerase or of the heat shock regions themselves.

It has been shown that there is an alteration in the micrococcal nuclease digestion pattern and an increase in the DNase I sensitivity of the heat shock loci upon activation (Wu et al., 1979). This increased DNase I sensitivity is typical of active genes, as shown by work in several other eukaryotic systems [e.g., Weintraub & Groudine (1976), Bellard et al. (1977), Garel et al., (1977), and Palmiter et al. (1977)].

Using nuclei prepared from heat-shocked embryos as described in this paper, we have observed (M. A. Keene, D. W. Miller, and S. C. R. Elgin, unpublished experiments) the same alterations in nuclease digestion patterns as those previously shown to occur at the heat shock loci (Wu et al., 1979). It has recently been reported that a 0.35 M NaCl wash of chromatin removes the DNase I sensitivity of transcribing genes in the chick red blood cell. This has been attributed to removal of the proteins HMG 14 and HMG 17, for upon their readdition to HMG-depleted chromatin, the sensitivity of active loci was restored (Weisbrod et al., 1980). In *Drosophila*, a protein fraction, band 2, has been identified which is released from nuclei upon DNase I digestion. Immunofluorescent labeling of polytene chromosomes, using antibodies directed against band 2, indicates that these proteins are preferentially associated with active loci and with loci scheduled to be active in the salivary gland (Mayfield et al., 1978). Recently, such a distribution pattern has been observed by using monoclonal antibodies against the same immunogen, indicating that this association is a property of a particular NHC protein of 62000 molecular weight (Howard et al., 1981).

Clearly then, there is evidence from several eukaryotic systems of proteins contributing to or, perhaps in and of themselves, conferring upon loci a transcriptionally active structural state. Such a mechanism seems likely to play a role in the heat shock response and could account for its stability. The band 2 protein fraction, for example, can be detected at heat shock loci after their induction by heat shock (Mayfield et al., 1978; Howard et al., 1981). The data presented in this paper indicate that those mechanisms which stabilize the induced heat shock state and promote the appropriate transcriptional responses are maintained in these isolated nuclei. One may infer that the template structure and enzyme activities required for specific transcription are present and representative of the cell nucleus at the time of isolation. This system should be of use in a continuing analysis of the process of gene activation and expression.

Acknowledgments

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