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Transcription of the Major *Drosophila* Heat-Shock Genes in Vitro†

Brian L. Craine and Thomas Kornberg*

ABSTRACT: Active eukaryotic genes are more accessible to some proteins that bind DNA than are inactive genes. In order to probe the accessibility of the *Drosophila* heat-shock genes, we have isolated nuclei from *Drosophila* tissue culture cells and have used these nuclei as templates for *Escherichia coli* RNA polymerase. With nuclei isolated from cells that had not been heat shocked, the synthesis of heat-shock RNA was not detected by hybridization to a DNA clone containing sequences from the major heat-shock region. In contrast, approximately 0.22% of the RNA synthesized in nuclei isolated from cells that had been previously heat shocked hybridized to the heat-shock clone. The synthesis of heat-shock RNA was DNA dependent, was sensitive to rifampicin and to actinomycin D, and represented a 70-fold enrichment over random transcription of the *Drosophila* genome. Transcription showed an extraordinary preference for a region 5′ distal to the structural gene. These results demonstrate that preferential transcription by the bacterial RNA polymerase is indicative of the active state of *Drosophila* genes.

Evidence from numerous sources has documented the structural differences between actively transcribed and inactive chromatin. In some cases, transcriptionally active or inactive chromatin can be distinguished by gross morphology. Three striking examples are the less compact chromatin associated with active genes in the lampbrush chromosomes of amphibian oocytes, in the ribosomal RNA genes of *Oncopeltus* (Foe, 1977), and in the giant puffs of the insect salivary gland chromosomes (Lamb & Daneholt, 1979). Enzymatic probes of chromatin structure have also indicated structural differences between active and inactive chromatin: active chromatin is more accessible to some proteins that bind DNA. DNase I preferentially digests globin genes that are active in erythrocyte nuclei but does not preferentially digest the inactive

† From the Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143. *Received April 24, 1981.* This work was supported by a grant from the National Institutes of Health (GM 25581) and a Faculty Research Award from the American Cancer Society.

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ovalbumin genes (Weintraub & Groudine, 1976; Gadski & Chae, 1978); ovalbumin genes are preferentially digested in oviduct nuclei where they are active (Garel & Axel, 1976). Active eukaryotic genes also appear to be more accessible to *Escherichia coli* RNA polymerase, as judged by their preferential transcription (Chiu et al., 1975; Paul, 1976; Tan & Mujager, 1970). The molecular basis for these functional differences is not understood as yet.

Expression of the *Drosophila* genome responds to environmental changes and does so dramatically in response to temperature elevation: a drastic reduction in the total number of RNA and protein products and a concomitant induction of approximately nine RNA and protein products ensue (Ashburner & Bonner, 1979). In this paper, we characterize the preferential transcription by *E. coli* RNA polymerase of the active heat-shock genes of *Drosophila melanogaster*. Using a cloned DNA probe, we have monitored the RNA made by *E. coli* RNA polymerase from the heat-shock gene that codes for the 70 000-dalton protein, the most abundant product of heat-shocked *Drosophila* cells. We report here that the *E. coli* RNA polymerase preferentially transcribes the major heat-shock gene after, but not before, activation.

Materials and Methods

Growth of Cells. The *Drosophila* K_c cell line established by Echalier & Ohanessian (1970) and obtained from Dr. Keith Yamamoto was used. The line was grown in D20 medium (Echalier & Ohanessian, 1970) without serum at 23–24 °C in spinner flasks. Cell number was maintained at (2–6) × 10^6 cells/mL.

Preparation of Nuclei. Cells were collected by centrifugation (either directly from the spinner flask or after being incubated at 35 °C for 25 min) and washed with HEES buffer [0.015 M *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.5), 60 mM KCl, 15 mM NaCl, 2.5 mM trisodium ethylenediaminetetraacetate (Na_3EDTA), 0.1 mM trisodium ethylene glycol bis(β-aminomethyl)ether-*N,N,N',N'*-tetraacetate (Na_3EGTA), and 0.25 M sucrose]. The pellet was resuspended to a density of 4 × 10^8 cells/mL and subjected to 1200 psi of nitrogen for 5 min in a Kontes pressure cell on ice. The nuclei in the effluent were pelleted, washed with HEES buffer, resuspended at 4 × 10^9 nuclei/mL, and stored on ice until used.

When appropriate, nuclei were resuspended for acid treatment in an acetate buffer (0.05 M sodium acetate, pH 3.0, 0.2 M KCl, 0.015 M NaCl, 2.5 mM Na_3EDTA, 0.1 mM Na_3EGTA, and 0.25 M sucrose) for 10 min on ice, then pelleted, washed with HEES buffer, and resuspended at 4 × 10^9 nuclei/mL.

RNA Synthesis in Vitro. Nuclei (4 × 10^7) were added to a reaction mixture of 0.015 M Hepes (pH 7.5), 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.25 M sucrose, 500 μM each of ATP, CTP, and GTP, 25 μM UTP, 0.015 mCi of [5,6-$^3H_2$]uridine 5'-triphosphate or 0.03 mCi of [α-^{32}P]uridine 5'-triphosphate, 0.1 mg/mL pyruvate kinase, and 12.5 mM phosphoenolpyruvate in a final volume of 0.1 mL. Reactions were carried out at 25 °C for 1 h. In experiments with *E. coli* polymerase (purified through the high-salt Bio-Gel A-5m column step according to Burgess & Jendrisak (1975)], 10 μg of the preparation was used.

Purification of RNA and Hybridization Conditions. Reactions were terminated by the addition of deoxyribonuclease I (30 μg/mL). After 2 min at 37 °C, samples were adjusted to 0.5% sodium dodecyl sulfate ($NaDodSO_4$), made 300 μg/mL in proteinase K, and incubated for 15 min at 37 °C. Sodium acetate (pH 5.0) was added to 0.2 M, and the RNA was precipitated with 2 volumes of ethanol at −80 °C.

For hybridization, the ethanol pellet was resuspended in 50 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) (pH 7.0), 0.6 M NaCl, 0.05 M Na_3EDTA, 0.01 M sodium pyrophosphate, and 60% formamide. The RNA, in a volume of 0.1 mL, was hybridized simultaneously to filters containing either plasmid 132E3 DNA or calf thymus DNA.

RNA probes for hybridization to restriction fragments were purified by hybridization to filters containing 132E3 DNA as described above. The RNA was recovered from the filter and hybridized to DNA fragments which had been transferred to nitrocellulose strips from an agarose gel according to the procedure of Southern (1975). This hybridization was accomplished in 60 mM Tes (pH 7.0), 0.75 M NaCl, 0.05 M Na_3EDTA, 16 mM sodium pyrophosphate, 0.2% $NaDodSO_4$, and 0.4 mg/mL yeast RNA in heat-sealable bags at 65 °C. The filters were repeatedly washed in 2 × SSC buffer (0.3 M NaCl and 0.03 M sodium citrate, pH 7.0) plus 1.0% $NaDodSO_4$ at 45 °C for 6 h. Bands were visualized by autoradiography using a Cronex intensifying screen.

Electrophoresis of DNA Fragments. Plasmid DNA was cleaved with the appropriate restriction endonuclease in cleavage buffer [6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 0.1 M NaCl, and 1 mM dithiothreitol] and loaded onto a horizontal 1% agarose gel in 20 mM sodium phosphate (pH 7.0) buffer. The running buffer was also 20 mM sodium phosphate (pH 7.0), and the gels were run at 130 V.

Preparation of Plasmid DNA. Plasmid 132E3 [kindly provided by Dr. Schedl (Schedl et al., 1978)] was prepared by precipitation of host chromosomal DNA with $NaDodSO_4$ and high salt according to Guerry et al. (1973). The supernatant containing the plasmid was extracted with phenol/chloroform (1:1) followed by several chloroform extractions. DNA was concentrated by ethanol precipitation. The concentration of plasmid DNA was determined by comparison of the fluorescence of ethidium bromide staining in an agarose gel with that of known concentrations of DNA.

DNA to be immobilized on a nitrocellulose filter was first denatured by heating at 100 °C for 3 min in 0.5 N NaOH. The solution was neutralized, adjusted to 4 × SSC, and filtered through Schleicher & Schuell BA85 filters. The filters were baked at 80 °C in a vacuum oven for 2 h.

Results

Transcription in Isolated Nuclei. Nuclei isolated from *Drosophila* tissue culture cells actively synthesize RNA when supplemented with ribonucleoside 5'-triphosphates (rNTP's). As judged by sensitivity to α-amanitin, about 20% of the RNA synthesis is catalyzed by RNA polymerase II. To investigate whether *E. coli* RNA polymerase can, when added to the nuclei, discriminate between active and inactive heat-shock genes, we reduced the background RNA synthesized by the endogenous *Drosophila* RNA polymerases. Brief exposure to acid pH has been found to inactivate the RNA polymerase activity in yeast nuclei (Tekamp et al., 1979), and a similar treatment of *Drosophila* nuclei is also effective (Table I). Treatment of nuclei at pH 3.0 and 200 mM KCl for 10 min at 0 °C reduced total synthesis by about 4-fold and essentially eliminated synthesis attributable to RNA polymerase II. The nuclei maintained their morphological integrity after these treatments.

Upon addition of exogenous *E. coli* polymerase, a high level of RNA synthesis was restored to acid-treated nuclei. Synthesis was proportional to the amount of polymerase added up to 20 μg of polymerase per 4 × 10^7 nuclei (Figure 1), indicating that the DNA template is in excess in this con-

Table I: Effect of KCl Concentration on in Vitro RNA Synthesis in Isolated Nuclei

	[KCl] (mM)	RNA synthesis rate [pmol (30 min)$^{-1}$ (2 × 10^7 nuclei)$^{-1}$] −α-amanitin	+α-amanitin[a]	total incorporation (%)	synthesis sensitive to α-amanitin (%)
acid-treated nuclei					
nuclei[b]	60	60.8	48.7	100	20
acid nuclei[c]	60	21.7	15.8	36	27
acid nuclei	150	17.8	17.2	29	3
acid nuclei	200	15.5	15.3	25	0
normal nuclei					
nuclei	60	56.0	43.1	100	23
nuclei	150	66.4	45.1	119	32
nuclei	200	53.6	40.2	96	25

[a] α-Amanitin was added to 5 μg/mL. [b] Nuclei were prepared in HEES buffer as described under Materials and Methods. [c] Nuclei were treated in pH 3 buffer as described under Materials and Methods at the indicated salt concentrations.

Table II: Heat-Shock Genes Preferentially Transcribed in Isolated Nuclei by *E. coli* RNA Polymerase

nuclei	RNAP[a]	α-amanitin (5 μg/mL)	RIF[b] (50 μg/mL)	ACT D[c] (50 μg/mL)	DNase I (30 μg/mL)	RNA synthesis (cpm)	hybridization to 132E3 DNA (cpm)	hybridization (%)
control nuclei	−	−	−	−	−	65 000	<10	<0.01
	−	+	−	−	−	56 000	<10	<0.01
control acid nuclei[d]	−	−	−	−	−	17 500	<10	<0.01
	−	+	−	−	−	17 200	<10	<0.01
	+	−	−	−	−	554 250	<10	<0.01
heat-shocked nuclei	−	−	−	−	−	57 240	500	0.90
	−	+	−	−	−	48 500	20	0.04
	+	+	−	−	−	254 800	444	0.17
heat-shocked acid nuclei	−	−	−	−	−	15 000	<10	<0.01
	+	−	−	−	−	334 400	740	0.22
	+	−	+	−	−	16 200	<10	<0.01
	+	−	−	+	−	35 000	<10	<0.01
	+	−	−	−	+	17 500	<10	<0.01

[a] *E. coli* RNA polymerase. [b] RIF represents rifampicin. [c] ACT D is actinomycin D. [d] Nuclei were treated with sodium acetate (pH 3) to inactivate endogenous RNA polymerase II as described under Materials and Methods.

centration range. Excess template should provide optimal conditions for utilizing polymerase as a probe for active genes.

RNA synthesis by the bacterial polymerase using acid-treated nuclei as template was linear for about 1 h (Figure 2) and continued for 2–3 h. Rifampicin reduced the synthesis to a level indistinguishable from that of the endogenous activity, indicating that the additional incorporation was due entirely to the bacterial polymerase. Therefore, the incorporation did not result from contaminating activities such as polynucleotide phosphorylase that might end-label preexisting molecules.

Hybridization Analysis of RNA Products. The nature of the RNA synthesized by isolated *Drosophila* nuclei was determined by hybridization to the plasmid probe 132E3. This plasmid is a genomic clone from 87C, the locus that codes for the major 70 000-dalton heat-shock protein (Schedl et al., 1978; Mirault et al., 1979). In an initial experiment, the RNA synthesized in vitro by endogenous polymerases was purified from nuclei isolated from control cells or from nuclei isolated from cells which had been heat shocked by incubation for 25 min at 37 °C. Hybridization of this RNA to excess 132E3 DNA revealed that control nuclei did not synthesize detectable amounts of heat-shock RNA (<0.01%). In contrast, nuclei from heat-shocked cells synthesized a significant amount of heat-shock RNA (see Table II). About 0.9% of the total RNA synthesized by heat-shocked nuclei hybridized to 132E3 DNA, indicating that 1.8% of the RNA synthesized was complementary to the major heat-shock gene (allowing for a hybridization efficiency determined to be 50%). Since only 15% of the total RNA synthesized was α-amanitin sensitive, approximately 12% of RNA synthesized by RNA polymerase II represents transcription of the major heat-shock gene. Synthesis of the heat-shock RNA was sensitive to low concentrations of α-amanitin, confirming that synthesis of this species is due to RNA polymerase II.

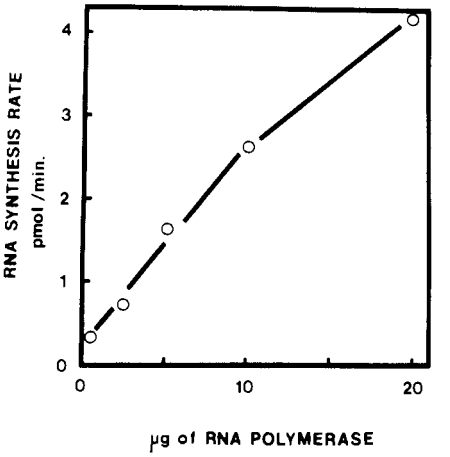

FIGURE 1: Dependence of the RNA synthesis rate on the concentration of RNA polymerase. The indicated amount of *E. coli* RNA polymerase was added to a standard reaction mixture containing 4 × 10^7 acid-treated nuclei. The amount of label incorporated in 20-μL samples was determined by acid precipitation as a function of time. From these kinetics, the initial rate of RNA synthesis was determined and is plotted as a function of the RNA polymerase concentration.

Acid-treated, heat-shocked nuclei did not support endogenous synthesis of heat-shock RNA. When *E. coli* RNA polymerase was added, nuclei isolated from control cells similarly did not synthesize heat-shock RNA. However, heat-shock

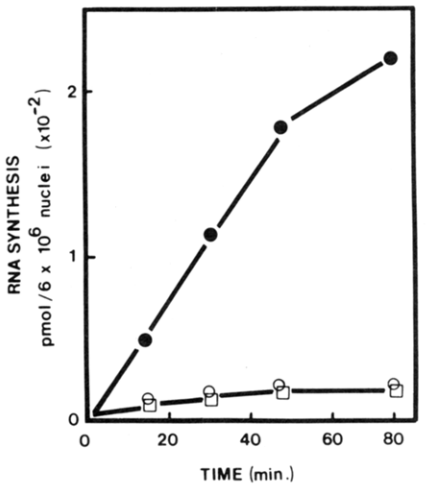

FIGURE 2: Kinetics of RNA synthesis using acid-treated nuclei as a template. Acid-treated nuclei isolated from K_c cells were added to standard reaction mixtures along with either 10 μg of RNA polymerase (●), 10 μg of RNA polymerase and 50 μg/mL rifampicin (□), or no additions (○). At the indicated times, the amount of label incorporated was determined by acid precipitation of 15-μL aliquots.

RNA was synthesized by *E. coli* RNA polymerase with acid-treated nuclei isolated from heat-shocked cells (Table II). Here, 0.22% of the total RNA synthesized hybridized to 132E3 DNA. Since the number of copies of this heat-shock gene per haploid genome is about 6 (Mirault et al., 1979) and the size of the *Drosophila* genome is about 340 000 kilobases (Kb), this amount of RNA complementary to the heat-shock region is about 70 times that expected from random transcription. This synthesis of hybridizable RNA was completely sensitive to rifampicin, confirming that endogenous activity had been eliminated and that the synthesis was catalyzed by the added *E. coli* RNA polymerase. Preferential transcription of the heat-shock gene region can also be observed in nuclei which have not been acid treated. The amount of heat-shock RNA synthesized by the bacterial polymerase can be measured by including α-amanitin to eliminate the endogenous polymerase II transcripts of the heat-shock genes. Table II demonstrates that the level of transcription of the heat-shock genes after acid treatment or administration of α-amanitin is indistinguishable.

The requirement for a DNA template was tested in two ways. First, inclusion of actinomycin D at 50 μg/mL reduced total RNA synthesis by 90% due to its intercalation into DNA, and no detectable amounts of heat-shock RNA were synthesized. Second, treatment of nuclei from heat-shocked cells with DNase I prior to transcription also eliminated the synthesis of heat-shock RNA (see Table II), showing that the RNA synthesized is DNA dependent and cannot represent an aberrant type of RNA-copying reaction.

Transcription Mapping. The regions of the *Drosophila* chromosome transcribed by *E. coli* RNA polymerase were analyzed by hybridization of RNA products to restriction fragments from plasmid 132E3 DNA by using Southern blotting (Southern, 1975). Cleavage of 132E3 with *Xba*I and *Sal*I endonucleases results in five restriction fragments: the largest contains the vector (about 12 kb); a long stretch of *Drosophila* DNA (about 3 kb) distal to the 5′ region that is transcribed in vivo (Figure 3) represents another fragment; an approximately 2-kb fragment represents about 85% of the structural gene; an approximately 0.78-kb fragment includes the space region between the genes; and finally, there is a small fragment from the 3′ end of the first gene of the repeat which is lost on this gel (Schedl et al., 1978). Thus, cleavage with

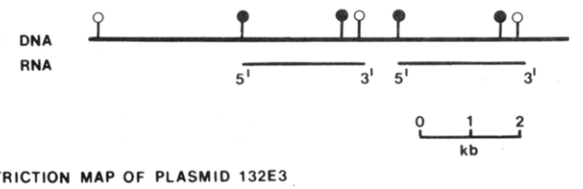

FIGURE 3: Restriction map of the *Drosophila* DNA fragment present in plasmid 132E3. The position and polarity of the messenger RNA for the 70K heat-shock protein are indicated. These data are taken from Schedl et al. (1978). *Xba*I (●); *Sal*I (○).

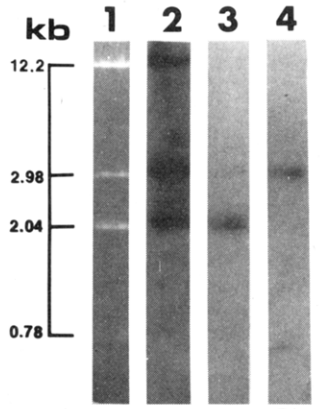

FIGURE 4: Transcription mapping of RNA synthesized in vitro. Plasmid 132E3 was cleaved with *Xba*I and *Sal*I in combination, and the fragments were resolved on an agarose gel. Lane 1 shows the ethidium bromide fluorescent staining marking the position of the DNA fragments in the gel. The DNA fragments were blotted onto nitrocellulose (Southern, 1975) and hybridized to RNA probes as described under Materials and Methods. The RNA probes used were cRNA prepared by using the *E. coli* RNA polymerase and plasmid 132E3 as a template (lane 2), RNA synthesized by endogenous polymerases by using heat-shocked nuclei (lane 3), or RNA synthesized with the *E. coli* RNA polymerase by using heat-shocked, acid-treated nuclei as a template (lane 4).

the *Xba*I and *Sal*I restriction endonucleases separates the coding sequence from the region predominantly on the 5′ side of the gene.

RNA synthesized by the endogenous RNA polymerase II in nuclei isolated from heat-shocked cells was hybridized to the restriction fragments. Hybridization was almost entirely to the 2-kb fragment containing the structural gene (Figure 4, lane 3). A small amount of hybridization was observed with the smallest fragment that contains portions of the gene. There was no hybridization to the DNA 5′ to the structural gene.

The RNA synthesized by the exogenous bacterial polymerase by using acid-treated nuclei from heat-shocked cells was tested. Essentially all of the hybridizable RNA synthesized was complementary to the 3-kb fragment in this experiment (Figure 4, lane 4). In some experiments, as much as 10% of the label has been observed to hybridize to the 2-kb structural gene fragment (not shown). The lack of labeling at the 2-kb fragment is not due to endogenous nonradioactive RNA competing out the new transcripts. This was determined by showing that the 2-kb fragment is available to hybridize cRNA to the same extent as control filters (unpublished results).

The role of the DNA sequence in specifying transcription by the bacterial polymerase can be determined by transcribing isolated plasmid DNA. RNA prepared in this fashion hybridized to all restriction fragments of plasmid 132E3. The selective transcription observed in lane 3 with endogenous polymerase II or in lane 4 with the bacterial polymerase is not observed when the purified DNA sequences are transcribed. This would indicate that on a gross level transcription of the *Drosophila* sequences by *E. coli* RNA polymerase is inde-

pendent of nucleotide sequence, with all regions of the insert having an equal potential for transcription.

Thus, the specificity of the bacterial polymerase differs from that of the endogenous polymerase II. A large proportion of the transcription by the bacterial polymerase derives from a region upstream from the structural gene and upstream from the endogenous population of RNA molecules. The pattern of transcription in isolated nuclei apparently is not determined solely by DNA sequence (compare with transcription of plasmid 132E3) but is probably influenced by DNA conformation or other factors.

Discussion

Active heat-shock genes in isolated nuclei are preferred templates for transcription by *E. coli* RNA polymerase: as much as 0.22% of the RNA synthesized by using heat-shocked nuclei as the template was found to be complementary to the heat-shock region represented on plasmid 132E3. Nuclei from cells which had not been heat shocked supported no synthesis of heat-shock RNA. These results indicate that hybridization analysis of the RNA synthesized by *E. coli* RNA polymerase assays the active state of the gene.

That the bacterial polymerase preferentially transcribes active regions of the chromosome has been established for over a decade (Smith et al., 1969; Tan & Mujager, 1970; Paul, 1976). Yet the interpretation of these experiments has been compromised by the possibility of the bacterial polymerase preparation labeling preexisting RNA molecules (Zasloff & Felsenfeld, 1977; O'Malley et al., 1978). End labeling or copying of preexisting mRNA molecules would be DNA independent and resistant to rifampicin, would be competed by the presence of the DNA template, and would hybridize to the same region of DNA as endogenous RNA molecules. If the transcription was at DNA sites primed by a preexisting mRNA, one would expect a rifampicin-sensitive, DNA-dependent reaction, and the resulting product would hybridize in the same region of DNA as endogenous RNA as endogenous RNA or to the 3′ side of the gene.

The results described here employed reaction conditions of excess template (see Figure 1) that reduced the likelihood of aberrant synthesis. The synthesis of hybridizable RNA is DNA dependent as demonstrated by sensitivity to DNase I and actinomycin D. The synthesis is also sensitive to rifampicin. Although the conditions of the reaction mixture [i.e., high nucleoside triphosphate concentrations (Di Nocera et al., 1975)] make an RNA-priming reaction unlikely, the fact that the synthesis occurs to a large extent on the 5′ side of the gene rules out that type of artifact. Thus, the characteristics of this RNA synthesis are consistent only with a typical DNA-directed *E. coli* polymerase reaction.

It is interesting that the region of the chromosome that is preferentially accessible to the bacterial polymerase appears to extend beyond the gene itself. As judged from the intensity of the autoradiograms in Figure 4, at least 90% of the RNA hybridizing to 132E3 was complementary to sequences 5′ distal to the structural gene. Although the structural gene itself is repeated about 6 times in the genome (Mirault et al., 1979), the sequences to the 5′ side are nonhomologous (Schedl et al., 1978). In the case of the fragments produced by *Xba*I and *Sal*I endonuclease double digestion, the ratio of the DNA mass for gene/5′ sequences would be (6 × 2.04):2.98 or 4.1:1.0. However, the ratio of RNA transcribed from these regions by the bacterial polymerase is at most 0.1:1.0 (Figure 4, lane 4), indicating that the 5′ prime region is a highly preferred site of RNA polymerase interaction. Although the molecular basis for the increased accessibility of the heat-shock chromatin to the *E. coli* polymerase is not known, the correlation of template availability with activation of the heat-shock locus and the striking preference for the chromatin 5′ to the active structural gene suggest a role in the regulation of expression. It is possible that the binding or removal of sequence-specific proteins changes the structure of the chromatin to permit an interaction between the polymerase and its promoter. It seems likely that some conformational change in the chromatin is monitored by the assay described here. In subsequent work, we have found that this assay can also monitor the in vitro activation of inactive heat-shock genes; a protein responsible for the in vitro activation has been purified and characterized (Craine & Kornberg, 1981).

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