Cell, Vol. 25, 671–681, September 1981, Copyright © 1981 by MIT

Activation of the Major Drosophila Heat-Shock Genes in Vitro

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Summary

Inactive Drosophila heat-shock genes of isolated diploid nuclei can be induced to a transcriptionally active state by exposure to cytoplasmic extracts from heat-shocked Drosophila cells. No effect was observed on histone gene transcription, and extracts from non-heat-shocked cells were ineffective. The factor in the cytoplasmic extract has been partially purified and characterized. It is protease-sensitive and heat-labile. A striking change accompanies in vitro activation that permits transcription by E. coli RNA polymerase of the chromatin 5′-distal to the structural genes at the 87A and 87C heat-shock gene loci; we have previously observed a similar change after in vivo heat-shock induction. That this change occurred in the absence of endogenous RNA polymerase II activity suggests that these changes may reflect the earliest event in gene activation. Inasmuch as activation also took place after histone H1 depletion, this histone does not appear to be essential for this step of gene activation.

Introduction

Chromatin is organized into a fundamental repeat unit, the nucleosome (Kornberg, 1974), yet the conformations of active and inactive genes differ. Decondensation accompanies active transcription of many chromosomal loci in insects (Foe, 1977; Lamb and Daneholt, 1979) and amphibians (Callan, 1966); structural modifications of active chromatin are revealed by observations such as increased sensitivity to deoxyribonuclease digestion (Weintraub and Groudine, 1976), preferential transcription by E. coli RNA polymerase (Tan and Mujager, 1970; Chiu et al., 1975; Paul, 1976; Craine and Kornberg, 1981) and association with the HMG class of proteins (Weisbrod et al., 1980). The finding of structural differences between inactive and actively transcribed chromatin does not necessarily mean that conformation regulates gene expression—the changes observed may be a consequence, rather than a cause, of active transcription. There is, however, electron microscopic evidence of changes in chromatin structure that precede transcription in the case of ribosomal genes in Oncopeltus (Foe, 1977). Moreover, genetic studies in Drosophila (Spofford, 1976) and in the mouse (Eicher, 1970) have shown that juxtaposition of an active euchromatic gene with highly condensed heterochromatin may inactivate the gene. These observations indicate that chromatin structure may be a determinant in gene regulation, but conclusive evidence can only come from studies at the molecular level.

We have investigated the molecular basis of the activation of the heat-shock genes of Drosophila. Drosophila cells respond to a temperature jump ("heat shock") from 25°C to 37°C with immediate changes in RNA synthesis, RNA processing and protein synthesis (Ashburner and Bonner, 1979). Of the nine newly synthesized RNA and protein products, the 70 kilodalton (kd) heat-shock protein is the most abundant, and its message accounts for a large fraction of the RNA synthesized during heat shock. Six copies of the gene coding for this peptide are present per haploid genome in Drosophila tissue-culture cells, and these repeated genes are distributed at two loci: 87A and 87C. Previous attempts to reconstruct the activation of the heat-shock genes in vitro have succeeded in inducing heat-shock puffs in the polytene chromosomes of isolated salivary gland nuclei (Compton and Bonner, 1977; T. Kornberg, unpublished results).

We describe here an in vitro system that reconstructs the activation of one of the gene loci induced upon heat shock of Drosophila cells. This system has provided a functional assay for the changes in chromatin that accompany activation and has permitted the partial purification and characterization of a protein fraction that specifically activates this heat-shock gene in vitro.

Results

To monitor the relative accessibility of the Drosophila heat-shock genes at 87A–87C to E. coli RNA polymerase, we hybridized RNA transcribed in vitro in the presence of ^{3}H-ribonucleoside triphosphates (NTPs) to the recombinant DNA heat-shock plasmid 132E3. This heat-shock plasmid contains two copies of the structural gene for the 70 kd heat-shock protein and the adjoining sequences (Mirault et al., 1979). Pseudodiploid nuclei from the permanent tissue-culture cell line, Kc, were prepared by nitrogen cavitation, and these nuclei were incubated for various times with a Kc cytoplasmic extract. The nuclei were reisolated by low-speed centrifugation and the structure of the 87A–87C heat-shock gene was probed with E. coli RNA polymerase. Figure 1B shows that in nuclei incubated with cytoplasmic extract isolated from Kc cells that had not been heat-shocked, less than 0.01% of the synthesized RNA was complementary to the heat-shock plasmid. However, in response to incubation with extract isolated from cells that had been subjected to heat shock, synthesis of RNA complementary to the heat-shock plasmid was observed. The similarity of this in vitro response to the behavior of nuclei isolated from heat-shocked cells (Craine and Kornberg, 1981) suggests that an alteration of the

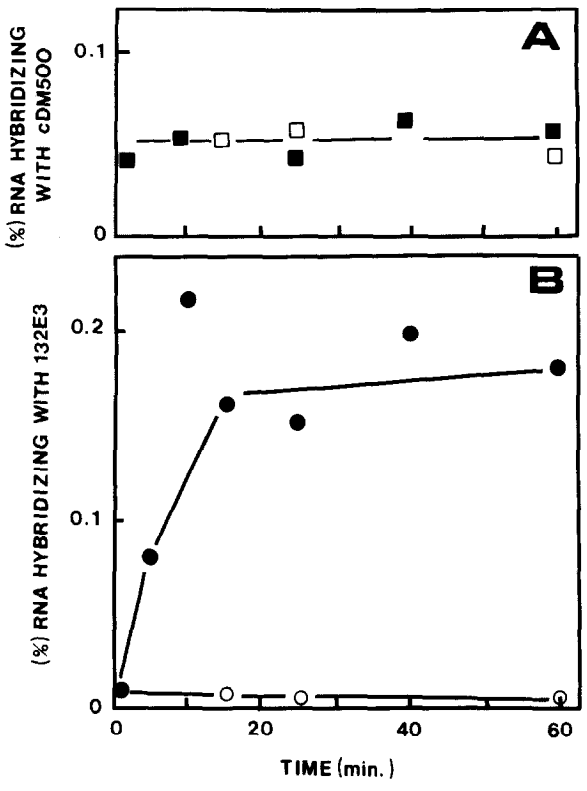


Figure 1. Kinetics of Gene Activation by Cytoplasmic Extracts

Nuclei isolated from Kc cells (10 μl) were mixed with 100 μl of cytoplasmic extract prepared from either heat-shocked Kc cells (solid symbols) or non-heat-shocked Kc cells (open symbols). Nuclei were then reisolated by centrifugation at the indicated times and were added to a transcription mixture containing bacterial RNA polymerase. The RNA synthesized was isolated and hybridized to a histone gene probe (cDM500) (squares in A) and to a heat-shock gene probe (plasmid 132E3) (circles in B).

chromatin template similar to that observed in in vivo gene activation has been catalyzed.

Several observations indicate the specificity of this in vitro activation: only extracts isolated from heat-shocked cells elicit the activation response (Figure 1); total incorporation of NTPs into acid-precipitable material was unaffected by incubations with extract (Table 1); the quantity of transcription complementary to the heat-shock gene region (0.18%) was much more than random transcription (<0.01%); and histone genes, which are highly repeated and are actively transcribed in the Kc cells, were transcribed by E. coli RNA polymerase (0.05%, as judged by hybridization to a genomic clone containing the histone genes), and this transcription was not affected by incubation with heat-shock extract (Figure 1A).

The dramatic and selective increase in RNA hybridizing to the heat-shock probe in response to incubation with heat-shock extract was proportional to the amount of extract added (see Figure 2B solid circles) in the range of about 0.05%–0.2% of the RNA transcribed. The largest fraction of RNA hybridizing to the heat-shock probe that has been observed was 0.4%. In contrast, there was no effect of increasing concentrations of heat-shock extract on the synthesis of RNA complementary to histone sequences (Figure 2A solid squares). Extract from non-heat-shocked cells had no effect on the synthesis of RNA complementary to either the heat-shock probe (Figure 2B open circles) or to the histone probe (Figure 2A open squares).

To confirm that the activation observed was in fact due to transcription of the DNA template by the exogenously added polymerase, we challenged the system in several ways (see Table 1). When the inhibitor of E. coli RNA polymerase initiation, rifampicin, was added during the transcription step at a concentration of 5 μg/ml, the total synthesis was reduced by 95% and the synthesis of heat-shock RNA was eliminated. This established that synthesis of heat-shock RNA was dependent on the bacterial polymerase. Actinomycin D (50 μg/ml), deoxyribonuclease and omission of nuclei each reduced total incorporation by over 90% and eliminated heat-shock RNA synthesis. These controls prove that the synthesis of the heat-shock RNA after activation is a typical DNA-dependent RNA polymerase activity.

Kinetics of Activator Formation in Vivo

Cells acquire activating capacity very rapidly in response to heat shock. However, their capacity is also lost rapidly after about 30 min, although some remains even after 4 hr. The amount of activating factor measured in this in vitro assay correlates with the relative size of the major larval salivary gland heat-shock puffs, which also reach a maximum size after a 30 min exposure (Figure 3).

Transcription Mapping of RNA Synthesized from Activated Nuclei

With nuclei isolated from heat-shocked cells, E. coli RNA polymerase preferentially transcribes a region to the 5′ side of the heat-shock structural gene rather than the gene itself (Craine and Kornberg, 1981). In contrast, the RNA synthesized by the endogenous polymerase II was complementary to the structural gene exclusively. In order to compare the accessibility of different portions of the heat-shock region in nuclei that had been activated in vitro or in vivo, RNA synthesized by the bacterial polymerase with nuclei as a template was hybridized to restriction fragments from the heat-shock plasmid. Digestion of plasmid 132E3 with Xba I and Sal I endonucleases produces five unique fragments (see Figure 4): a 2.98 kb fragment containing just 5′-distal sequences of one gene repeat and a 0.78 kb fragment containing 5′-distal sequences from the second gene repeat; fragments of 2.04 kb and 0.17 kb from the structural genes; and a 12.2 kb fragment containing vector sequences. The 0.17 kb fragment is not visualized in this gel. As seen in Figure 4A, activation of the nuclei in vivo or in vitro

Table 1. Heat-Shock RNA Is Synthesized by the Bacterial RNA Polymerase in a DNA-Dependent Fashion

Components	RNA Synthesis (pmole per assay)	Hybridization to 132E3 (cpm)	Hybridization (%)
Complete system[a]	1350	3200	0.24
+ Rifampicin (5 μg/ml)	59	<10	<0.01
+ Actinomycin D (50 μg/ml)	140	<10	<0.01
+ Deoxyribonuclease (20 μg/ml)	65	<10	<0.01
− Nuclei	9	<10	<0.01
− E. coli RNA polymerase	62	<10	<0.01
− Extract	1270	<10	<0.01

[a] Complete system is a standard assay of 100 μl of extract, as described in Figure 2. Nuclei were exposed to extract for 25 min, repurified by centrifugation and transcribed by RNA polymerase in the presence or absence of drugs and deoxyribonuclease, as indicated.

results in a pattern of RNA synthesis by the bacterial polymerase that is indistinguishable by this analysis (Figure 4A lanes 4 and 5). Most of the RNA from heat-shocked nuclei hybridizes to the 2.98 kb fragment that is 5′-distal to the first 70 kd gene, and less than 10% hybridizes to the 2.04 kb structural-gene fragment. No hybridization to the 0.78 kb fragment, 5′-distal to the second gene, was observed. In contrast, the endogenous RNA polymerase produces RNA that is predominantly homologous to the structural gene (Figure 4A lane 3). Figure 4A lane 6 demonstrates that the structural gene was available for hybridization under the conditions employed for the analysis of the RNA synthesized by the bacterial polymerase: cRNA prepared with plasmid 132E3 hybridizes to the structural gene fragment with equal efficiency before (lane 2) or after (lane 6) hybridization to the bacterial RNA transcripts displayed in lane 5.

To determine if the change in chromatin structure occurs at the 5′ side of other heat-shock genes, we examined sequences flanking a heat-shock structural gene at 87A. The plasmid 56H8 contains a genomic insert from this region of the chromosome and contains one copy of the same heat-shock gene present at 87C; the flanking sequences of this repeated gene family are nonhomologous (Mirault et al., 1979). Digestion of this plasmid with Xba I produces three fragments: a 2.04 kb structural-gene fragment, a 1.44 kb fragment containing 5′-distal sequences only and a fragment containing the vector plus some 3′ and additional 5′ sequences (Figure 5). Whereas the endogenous Drosophila RNA polymerase transcribed exclusively from the structural gene (Figure 5A lane 3), transcription of activated nuclei with exogenous bacterial polymerase produced RNA that hybridized primarily to the 5′-distal sequences and to the vector-containing fragment. This result is consistent with transcriptional patterns at the 87C locus and shows that when activated, at least two of the six copies of the heat-shock gene coding for the 70 kd protein show heightened accessibility to exogenous polymerase at the 5′ side of the gene.

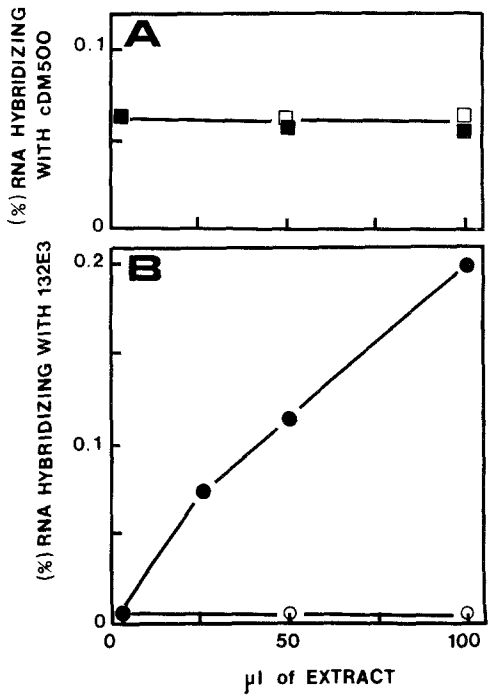


Figure 2. Dependence of Gene Activation on Concentration of Extract

Nuclei isolated from Kc cells (10 μl) were mixed with different concentrations of either heat-shock cytoplasmic extract (solid symbols) or cytoplasmic extract isolated from non-heat-shocked cells (open symbols) in a final volume of 0.1 ml. After 25 min of incubation at 25°C, the nuclei were reisolated and added to a reaction mixture containing bacterial polymerase. The RNA synthesized during a 1 hr incubation at 25°C was purified and hybridized to plasmid cDM500 (squares in A) and to plasmid 132E3 (circles in B).

An alternative explanation for the RNA hybridizing 5′-distal to the genes from both 87A and 87C is that the bacterial polymerase has a very high affinity for small regions referred to as the x and y elements (Moran et al., 1979), which are located just 5′ to the coding region. We tested this possibility by determining whether the RNA hybridizing to the 5′-distal regions of plasmid 132E3 crosshybridized with plasmid 56H8. The y elements in front of the repeating genes

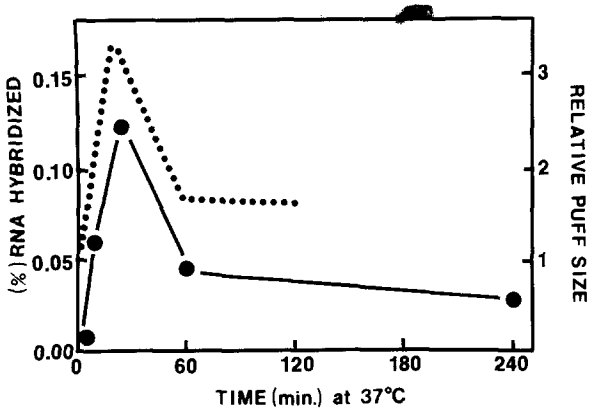
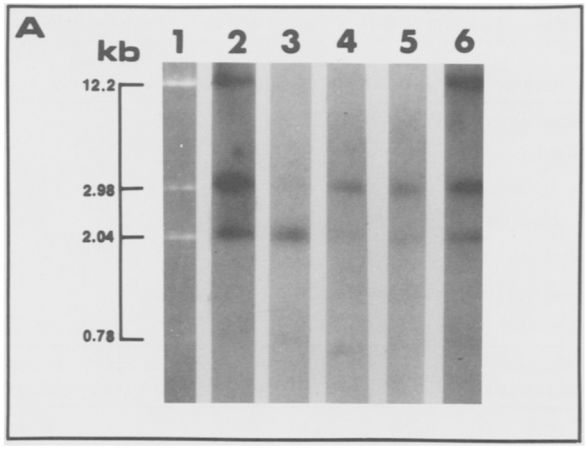


Figure 3. Kinetics of Production of Activator during Heat Shock

Kc cells were brought to 37°C rapidly in a 55°C water bath. At the indicated times (●—●) 100 ml of cells were sampled and an extract was prepared. We determined the amount of activator present by incubating nuclei in 100 μl of extract as described in Figure 2 and determining the fraction of RNA hybridizing to the heat-shock probe (plasmid 132E3). Also shown for comparison are data of Ashburner (1970) on the relative puff size of the 87C locus (dotted line) during the course of heat shock of salivary glands.



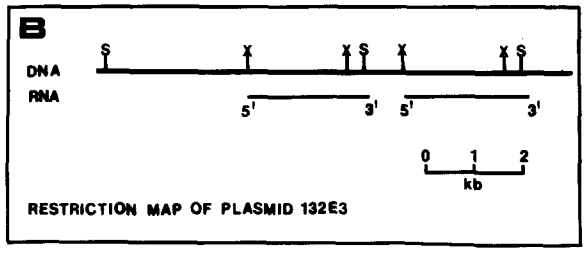


Figure 4. Transcription Mapping of RNA Transcripts to Restriction Fragments of the Heat-Shock Gene in Plasmid 132E3

(A) Plasmid 132E3 was digested by Xba I and Sal I endonucleases in combination and the resulting fragments were resolved on a 1% agarose gel. The DNA bands revealed by ethidium bromide fluorescence are seen in lane 1. The DNA fragments were blotted onto nitrocellulose and hybridized to RNA probes. Various RNA probes used were: cRNA prepared with E. coli RNA polymerase and plasmid 132E3 as a template (lanes 2 and 6), RNA synthesized by the endogenous polymerase in nuclei isolated from heat-shocked Kc cells (lane 3), RNA synthesized in vitro by the E. coli RNA polymerase with acid-treated nuclei isolated from heat-shocked cells as template (lane 4) and RNA synthesized by the E. coli RNA polymerase with nuclei activated by heat-shock cytoplasmic extract as template (lane 5). Note the random transcription of plasmid DNA (lane 2) but the selective transcription of chromatin by the Drosophila (lane 3) and E. coli (lanes 4 and 5) RNA polymerases. The filter used in lane 6 was the same filter used in lane 5, without further washing before hybridizing with cRNA.

(B) A restriction map of the Drosophila sequences present in plasmid 132E3. The positions of the mRNA of the duplicated genes and the polarity of the message and sites for Xba (X) and Sal I (S) restriction endonucleases are indicated. These data are from Mirault et al. (1979).

on plasmid 132E3 are similar, but different from the y element on plasmid 56H8. The x element on plasmid 56H8, however, is homologous to the x element in front of the first of the repeated genes on plasmid 132E3. RNA synthesized with nuclei prepared from heat-shocked cells as template was isolated by filter hybridization to plasmid 132E3. RNA was eluted and hybridized to restriction fragments from both plasmids 132E3 and 56H8 according to the method of Southern (1975) (Figure 6A lanes b and c, respectively). The nuclei had not been acid-treated and the endogenous polymerases therefore synthesized heat-shock RNA. This RNA served as an internal hybridization control. Figure 6A shows that the only region that crosshybridizes is the region containing the structural gene, while the fragments containing x and y elements from 56H8 (fragment III) show no detectable hybridization. The x-containing fragment from 132E3 (fragment I) does hybridize RNA. This result does not rule out the possibility that the E. coli polymerase might be initiating at the x element, but it does support the contention that the genes from both the 87A and 87C locus are being activated in vitro.

Transcript Length

We determined the length of the RNA transcripts produced by the E. coli RNA polymerase using activated nuclei as a template. After incubation of the nuclei with RNA polymerase the RNA products were purified by phenol:chloroform extraction. The RNA present in the activated nuclei without transcription by the bacterial polymerase was also purified for comparison. The RNAs were denatured, displayed on an agarose gel and transferred to a nitrocellulose filter according to the procedure of Thomas (1980). Hybridization with radioactive plasmid 132E3 DNA reveals the presence of a RNA band present in the heat-shock extract (Figure 7 lane a) migrating with an apparent size of 2500 bases. This value is close to the expected size for the mRNA of the 70 kd heat-shock protein. The sample with the transcripts from the bacterial polymerase (Figure 7 lane b) shows additional RNA homologous to the plasmid 132E3 with a size range from 100 to 900 bases in length. No discrete length of transcripts was found.

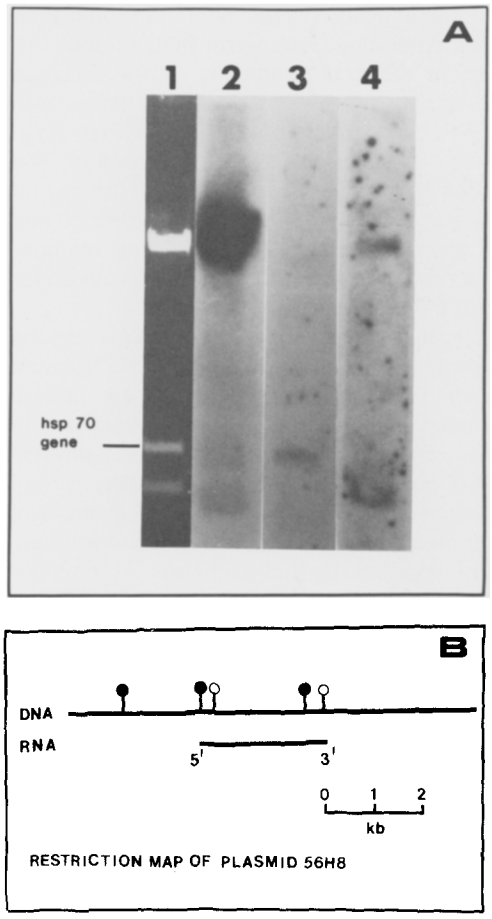


Figure 5. Transcription Mapping of RNA Transcripts to Restriction Fragments of Plasmid 56H8

(A) Plasmid 56H8 was hydrolyzed by Xba I endonuclease and the resulting DNA fragments (visualized by ethidium bromide staining; lane 1) were resolved on a 1% agarose gel. The fragments were transferred to nitrocellulose filters by Southern blotting (Southern, 1975) and hybridized to RNA probes. The RNA probes used were: cRNA prepared with E. coli RNA polymerase and plasmid 56H8 as template (lane 2), RNA synthesized by the endogenous polymerase in nuclei isolated from heat-shocked cells (lane 3) and RNA synthesized by E. coli polymerase with nuclei that had been activated by cytoplasmic extract from heat-shocked cells (lane 4). Transcription of purified plasmid 56H8 DNA showed some preference for the largest fragment (lane 2), and this preference might indicate the presence of a fortuitous strong promoter for the bacterial RNA polymerase in either the 5′ or 3′ flanking sequences. Since no such preference was observed between the small 5′ 1.44 kb fragment and the 2.04 kb gene fragment, whereas a distinct preference for the 1.44 kb fragment in activated nuclei was evident (lane 4), the asymmetry of transcription of these regions cannot derive simply from the DNA sequence.

(B) A restriction map of the Drosophila sequences present in plasmid 56H8. The position and polarity of the messenger RNA coding for *hsp70* and the cleavage sites for the Xba I (♀) and Sal I (♀) restriction endonucleases are indicated. These data are from Mirault et al. (1979).

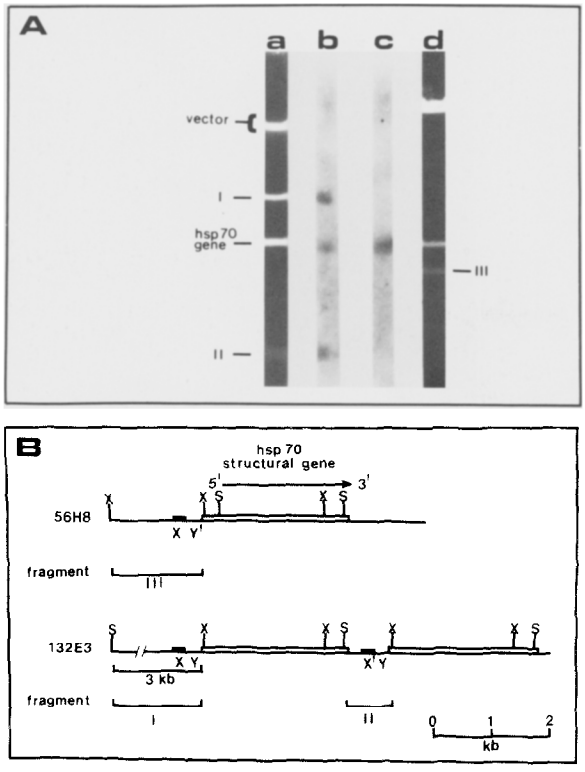


Figure 6. Determination of the Amount of RNA That Hybridizes to 132E3 and 56H8

(A) Plasmid 132E3 was digested with Xba I and Sal I in combination; plasmid 56H8 was digested with Xba I. The resulting fragments were resolved on a 1% agarose gel; the ethidium bromide staining pattern for 132E3 (lane a) and 56H8 (lane d) is shown. The fragments were transferred to nitrocellulose and hybridized with radioactive RNA that had been previously purified by filter hybridization to plasmid 132E3. The RNA was synthesized by E. coli RNA polymerase in vitro, with nuclei isolated from heat-shocked cells as a template. The endogenous polymerase was not inactivated, and therefore contributes radioactive heat-shock RNA complementary to the *hsp70* gene fragment as an internal hybridization control. (Lane b) hybridization of RNA to the 132E3 fragments; (lane c) hybridization to the 56H8 fragments.

(B) Restriction maps of the Drosophila DNA present in plasmids 56H8 and 132E3. Open bars: positions of the heat-shock structural genes that are homologous. Solid bars: x elements. The y elements are located between the x elements and the structural gene. The elements x′ and y′ are not homologous to the other x and y elements. Fragment I is 5′-distal to the first gene repeat on plasmid 132E3 and contains an x element homologous to the one present on fragment III from plasmid 56H8. Fragment II is the DNA spacer region between the repeats present on 132E3. X: Xba I. S: Sal I. This map is from Moran et al. (1979).

Partial Purification and Characterization of Activator

Cytoplasmic extract prepared as described in Experimental Procedures contains the activator in a particulate form. A particulate fraction of Kc cytoplasm prepared by centrifugation of the extract at 100,000 × g for 1 hr retains 91% of the activity in the pellet fraction and none in the supernatant. Increased ionic strength (2 M NaCl, 3 M KCl or 2 M $(NH_4)_2SO_4$), even in the presence of sodium deoxycholate and Triton X-100, failed to solubilize the activity, although some enrichment in the pellet was obtained.

To purify the activator, we adjusted the cytoplasmic extract to concentrations of 0.5 M KCl, 0.02 M sodium EDTA, 0.2% sodium deoxycholate and 0.2% Triton X-

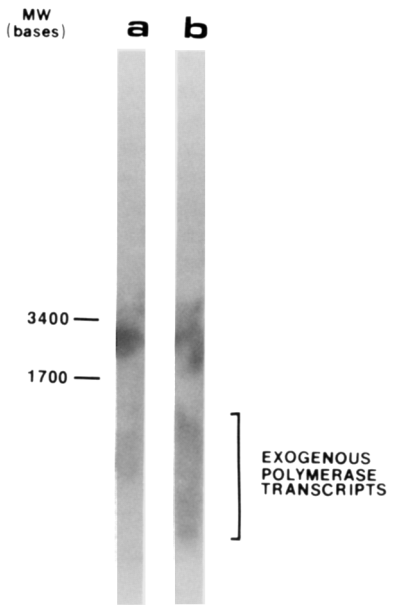


Figure 7. Molecular Weight of RNA Transcripts

Nuclei, prepared as described in the Experimental Procedures, were activated by exposure to heat-shock extract for 25 min. The nuclei were pelleted and resuspended in the transcription mixture without radioactive nucleotide triphosphates. A portion had E. coli RNA polymerase added and the nuclei were incubated at 25°C for 1 h. The RNA was purified and denatured according to the method of Thomas (1980) and displayed on a 1% agarose gel. E. coli ribosomal RNA was run in an adjacent well as a molecular weight standard. The RNA was transferred to nitrocellulose as described in Experimental Procedures and hybridized with nick-translated 132E3 DNA. (Lane a) RNA present in the activated nuclei preparation; (lane b) RNA present after transcription with the E. coli RNA polymerase.

100, centrifuged the extract at 20,000 × g for 10 min and layered it on a discontinuous sucrose gradient containing a 2 M sucrose shelf. The gradient was centrifuged at 100,000 × g for 1 hr and the fraction sedimenting to the shelf was recovered. This "DOC fraction" was enriched in dissociated ribosomes and had an A_{260}/A_{280} ratio of 1.7. The material that pelleted through the shelf included ribosomes, as determined by the presence of rRNA. The distribution of activity showed about 60% in the DOC fraction and about 10% in the pelleted fraction; the supernatant did not contain detectable amounts of activity.

The nature of the activator was characterized by its relative sensitivity to various treatments. The DOC fraction incubated at 100°C for 3 min lost all capacity to activate nuclei; the DOC fraction was also sensitive to ethanol precipitation and to phenol:chloroform extraction. Nuclei treated with any of the inactivated DOC fractions synthesized no detectable amounts of heat-shock RNA, compared with a level of 0.25% synthesized by a nontreated DOC fraction (Table 2A). The protein nature of the activator was further indicated by treatment of the DOC fraction with an immobilized protease (10 to 20 min at 25°C [Table 2B]). After protease was removed by filtration through glass wool, the activation capacity of the proteolyzed fraction was determined. A control DOC fraction, incubated under identical conditions with a cellulose matrix but without protease, induced an increase in heat-shock RNA synthesis by the E. coli RNA polymerase up to 0.25% of the total incorporation. The DOC fraction treated with protease for 10 min had only a very slight stimulating activity, and after 20 min there was no detectable activity remaining. We conclude that a protein in the DOC fraction is required for activation of the heat-shock genes.

High-salt washes were ineffective in solubilizing the activator protein from the particulate DOC fraction; however, washing the DOC fraction with 4 M urea in ammonium sulfate was effective. Optimal recovery of activity was obtained at an $(NH_4)_2SO_4$ concentration of 0.25 M (Figure 8). The activator, solubilized from the DOC fraction by 4 M urea and 0.25 M ammonium sulfate and recovered in a 100,000 × g supernatant, can be fractionated by ion-exchange chromatography.

Heat-Shock RNA Is Absent from Purified Fractions

The presence of heat-shock RNA in the activating fractions was determined by spot hybridizations (Thomas, 1980). Equal amounts of cytoplasmic extract from control cells, from heat-shocked cells and from the DOC and urea-solubilized fractions (equivalent in amount to the activating potential of the heat-shock extract) were phenol:chloroform-extracted. The purified nucleic acids were spotted on nitrocellulose and hybridized with radioactive plasmid 132E3. As shown in Figure 9, the control cytoplasmic extract showed no hybridization, while heat-shock RNA was present in the extract from heat-shocked cells. The DOC fraction had been treated with high salt, EDTA and detergents to release the heat-shock RNAs and, as expected, this fraction and the subsequent urea-solubilized fraction did not contain detectable amounts of heat-shock RNA.

Endogenous RNA Polymerase II Is Not Required for Activation

The nature of the modification of the chromatin that allows preferential transcription by the bacterial RNA polymerase is not understood. Since it is possible that transcription of that region of the chromosome by the endogenous Drosophila RNA polymerase II alters the chromatin structure and enables preferential transcription by the bacterial polymerase, it was of interest to evaluate the role of RNA polymerase II in this gene activation. Isolated nuclei were mixed with heat-shock extract in the presence or absence of α-amanitin (10 μg/ml) and were transcribed by the E. coli RNA polymerase in a standard assay. α-Amanitin had essentially no effect on activation of the heat-shock region (Table 3), implying that Drosophila RNA polymerase II activity is not required for activation. The

Table 2. Protein-Like Properties of Activating Factor

(A) Physical Inactivation of DOC Fraction

Treatment	RNA Synthesis (pmole UTP)	Hybridization to 1323E (cpm)	Activity Remaining (%)
None	2.2	2860	100
Boiled for 3 min	2.3	<10	<1
Phenol:chloroform extraction[a]	2.5	<10	<1
Ethanol precipitation	2.3	<10	<1

(B) Protease Digestion of DOC Fraction

Incubation at 25°C (min)	Immobilized Protease[b]	CM-cellulose Only	RNA Synthesis (pmole UTP)	Hybridization to 1323E (cpm)	Activity Remaining (%)
0	–	–	1.86	2090	100
10	–	–	1.90	2030	95
20	–	–	1.70	1920	100
10	+	–	1.73	370	12
20	+	–	2.11	<10	<1
20	–	+	1.50	1840	110

[a] The DOC fraction was extracted with an equal volume of phenol:chloroform (1:1) and then extracted three times with chloroform alone. This was followed by two ether extractions and exposure to a stream of nitrogen for 5 min.
[b] Streptomyces griseus protease immobilized on CM-cellulose (Sigma).

presence of α-amanitin during the transcription step by the bacterial polymerase also did not interfere with activation.

Other treatments that inactivate RNA polymerase II, concentrations of Mn^{2+} as low as 5 mM (Greenleaf and Bautz, 1975) and acid pH (Tekamp et al., 1979), similarly do not affect activation. Nuclei incubated for 25 min with heat-shock extract containing up to 30 mM Mn^{2+} can be activated by the heat-shock extract and show no effect of the inclusion of Mn^{2+} (Table 3). Similarly, nuclei that had been treated with acid could be activated to support the synthesis of heat-shock RNA (0.15% of the RNA synthesized by the acid-treated nuclei hybridized to the heat-shock plasmid [Table 3]). Thus RNA polymerase II is not required for activation.

Histone H1 Is Not Required for Activation

Acid-treated nuclei also afford an opportunity to investigate the role of histone H1, a protein believed to be involved in the higher order folding of chromatin (Kornberg, 1974), and therefore with a possible role in regulating the accessibility of chromatin. Acid-treated nuclei lose essentially all of the H1, as judged by a display of the nuclear proteins on an SDS-polyacrylamide gel (Figure 10; the loss of H1 after acid treatment was the only obvious change between the protein patterns of normal nuclei and of acid-treated nuclei). Since, as described above, acid-treated nuclei are capable of activation by heat-shock extract to the same extent as normal nuclei (see Table 3), we conclude that histone H1 is probably not participating in the activation.

Discussion

We have demonstrated elsewhere that preferential transcription by E. coli RNA polymerase can differentiate between an active or inactive heat-shock gene in nuclei isolated from Drosophila tissue-culture cells (Craine and Kornberg, 1981). It is here demonstrated that cytoplasmic extracts from heat-shocked cells are capable of conferring the characteristics of active genes on inactive heat-shock genes in nuclei isolated from non-heat-shocked cells. This in vitro "gene activation" specifically activates the heat-shock genes without affecting transcription of the histone genes; is catalyzed by a protein; is independent of endogenous RNA polymerase II activity; and does not require the presence of the histone H1. Our use of E. coli RNA polymerase differs significantly from previous investigations that have used this enzyme to monitor the transcription of eucaryotic genes. Our assay employs optimal conditions for discrimination by the bacterial polymerase, with template excess and saturating concentrations of nonmodified nucleotides, and does not require specific initiation. Transcription is monitored by hybridization to a genomic clone containing structural-gene as well as flanking sequences. The transcription is DNA-dependent and is sensitive to rifampicin and actinomycin D at concentrations that inhibit the normal activity of the enzyme. While the basis for the highly preferential transcription of the activated heat-shock gene region (70-fold higher than random transcription) is not understood, these studies demonstrate that with respect to quantity and specificity, the E. coli RNA polymerase transcripts of nuclei iso-

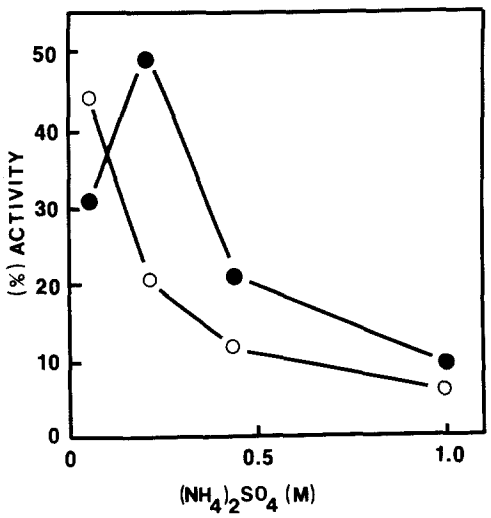


Figure 8. Solubilization of the Activating Protein by Urea and Salt Treatment

The DOC fraction was adjusted to 4 M urea and the indicated concentration of ammonium sulfate. After 10 min at 4°C, samples were centrifuged at 100,000 × g for 60 min in an SW65 rotor at 4°C and the resulting pellet was resuspended to the original volume. The supernatant (●——●) and the pellet (○——○) were assayed for the activating protein as described in Figure 2.

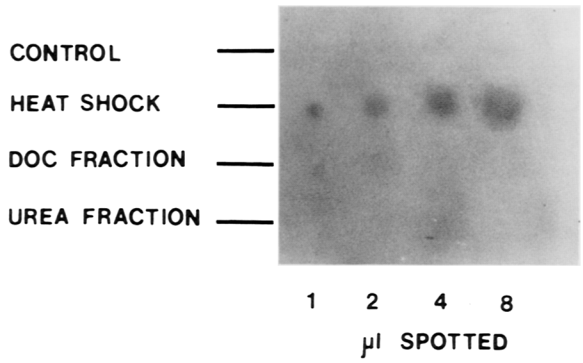


Figure 9. Determination of Heat-Shock RNA Content in Activating Fractions

Nucleic acids were purified from control cytoplasmic extract, heat-shock cytoplasmic extract and amounts of DOC and urea-solubilized fractions that activate to the same extent as the heat-shock extract. The indicated volumes of nucleic acids were spotted onto nitrocellulose and hybridized with nick-translated 132E3 DNA according to the procedure of Thomas (1980).

lated after in vivo heat shock or in vitro activation were indistinguishable. These properties were exploited to devise a complementation assay allowing for partial purification of the protein responsible for heat-shock gene activation.

Although any enzymatic activity associated with the activating protein remains unknown, its very tight binding to a large particle in cytoplasmic extracts suggests that its interaction with the heat-shock chromatin may not be direct. The purification of the activating protein has not resolved more than a single factor required for the activation of the isolated nuclei, but the impurity of these nuclei leaves the possibility that one or several intermediates carry the heat-shock response to the appropriate chromosomal loci. Such a mechanism offers one explanation for the measurable kinetics of the activation step (Figure 1). The observation that the heat-shock loci are induced even in the absence of protein synthesis (Ashburner, 1970) suggests that proteins involved in activation are components of cells during normal growth but are modified or translocated, or both, after heat shock. It therefore remains possible that the activating protein identified by the assay described here does not reflect the initial response of a heat-shocked cell. The delineation of the actual process of induction of the heat-shock loci and the identification of the components involved will require the further purification of this in vitro system.

The physiological basis for the heat-shock response is unknown, and its elucidation may require the identification of the enzymes induced upon heat shock. Possible alternatives include metabolic imbalance resulting from the modification of an essential component, or a normal response to a change in metabolic rate. It is hoped that the isolation and characterization of the activating protein will yield clues to the physiology of the heat-shock response; properties of the activating protein that have been revealed by its purification to date have yet to indicate whether the protein is bound to a ribosomal subunit or to some other large particle, and whether this binding occurs artifactually during lysis. But the possible association with ribosomes is intriguing, since the ribosome is a vital monitoring and control center in the cell and the heat-shock response bears some basic similarities to the stringent response of E. coli (Nierlich, 1978).

Our demonstration that the primary regulation of the heat-shock response is independent of RNA polymerase II does not eliminate the possibility that the specificity of the polymerase is altered during heat shock. RNA synthesized in cells during heat shock differs from RNA synthesized under normal growth conditions primarily in the RNA polymerase II transcripts. This may suggest that RNA polymerase II recognition has been altered during heat shock to increase its affinity for the heat-shock promoters while decreasing its affinity for other sites. Certainly the regions transcribed under heat-shock conditions by the E. coli polymerase and by the Drosophila polymerase differ markedly. However, the lack of requirement for the RNA polymerase would suggest that the control of gene transcription is primarily effected by factors that induce local changes in the chromatin, perhaps allowing for a more passive polymerase interaction. Transcription rate could be governed by the extent of the local conformational changes and promoter efficiencies.

Accessibility of the heat-shock gene region to E. coli polymerase increased after either in vivo (Craine

Table 3. Heat-Shock Gene Activation Does Not Require RNA Polymerase II Activity

	Nuclei	Heat-Shock Extract	α-Amanitin during Activation	α-Amanitin during Transcription	Mn^{2+} (mM)	Hybridization to 1323E3 (%)
Resistance of activation to α-amanitin (10 μg/ml)	Normal	−	−	−		<0.01
	Normal	+	−	−		0.095 ± 0.007
	Normal	+	+	−		0.070 ± 0.035
	Normal	+	−	+		0.087 ± 0.002
Resistance of activation to Mn^{2+}	Normal	+			0	0.14
	Normal	+			5	0.16
	Normal	+			10	0.13
	Normal	+			30	0.14
Activation of acid-treated nuclei	Acid-treated	−				<0.01
	Acid treated	+				0.15

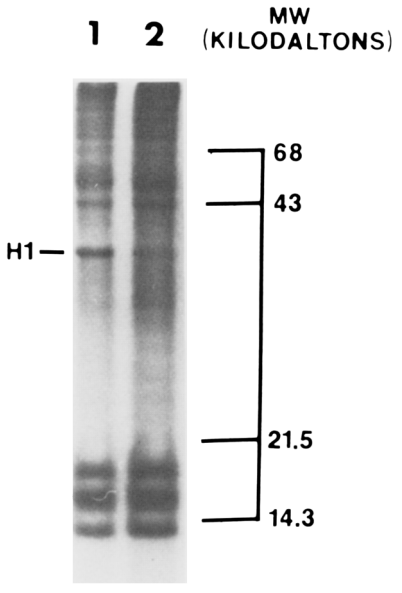


Figure 10. Effect of Acid Treatment on Nuclear Proteins

Nuclei were prepared from Kc cells by nitrogen cavitation. The protein patterns were determined by SDS-polyacrylamide gel electrophoresis in a 15% gel, as described in Experimental Procedures, before (lane 1) and after (lane 2) acid treatment. The position of histone H1 is indicated.

and Kornberg, 1981) or in vitro activation. We presume that this reflects a similar change in accessibility to the endogenous enzyme in vivo, and the location of this region of heightened accessibility, at the 5′ side of at least two of the genes, suggests its involvement in the control of expression. Although the molecular basis for the preferential transcription by the bacterial polymerase of the region 5′-distal to the structural gene is not understood, peculiarities in the base sequence of the region cannot be the cause: the pattern of transcription of naked plasmid DNA showed no such asymmetry.

Differential accessibility suggests instead, but does not prove, that changes in chromatin conformation have taken place. For instance, the increased accessibility of the bacterial polymerase might result from a reduction in nucleosome density. In Oncopeltus (Foe, 1977), in Chironomus (Lamb and Daneholt, 1979) and in the silk moth (McKnight et al., 1976), some actively transcribed genes have been observed to have fewer nucleosomes per unit length of DNA. Lamb and Daneholt (1979) found in addition that active BR2 chromatin has a region of DNA (about 500 bp) 5′-distal to the structural gene that is devoid of nucleosomes. Such a region preceding the heat-shock genes may offer a preferred site of transcription for E. coli RNA polymerase. Another possible explanation is that the removal of sequence-specific DNA-binding proteins could open a region to transcription by the bacterial polymerase without altering the folding of the chromosomal DNA.

In conclusion, we have shown that the changes that characterize the activation of heat-shock genes can be reproduced in vitro in the absence of gene expression and that a protein responsible for this induction can be identified. We suggest that these studies have also identified an alternative state of chromatin that may or may not be directly correlated with differential sensitivity to deoxyribonuclease digestion.

Experimental Procedures

Growth of Cells

We used the Drosophila Kc cell line established by Echalier and Ohanessian (1970) and obtained from K. Yamamoto. The line was grown in D20 medium without serum at 23–24°C in spinner flasks. Cell number was maintained at 2 to 6 × 10^6 cells/ml.

Preparation of Nuclei and Cytoplasmic Extract

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 mM HEPES [pH 7.5], 60 mM KCl, 15 mM NaCl, 2.5 mM sodium EDTA, 0.1 mM sodium EGTA and 0.25 M sucrose). The pellet was resuspended to a density of 4 × 10^8 cells/ml and was subjected to 1200 psi nitrogen for 5 min in a Kontes pressure cell on ice. The nuclei in the effluent were pelleted, washed with HEES buffer, resuspended at a density of 4 × 10^9 nuclei/ml in HEES buffer and stored on ice until used.

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 sodium EDTA, 0.1 mM sodium EGTA and 0.25 M sucrose) for 10 min on ice and then pelleted, washed with HEES buffer and resuspended at a density of 4 × 10^9 nuclei/ml.

We prepared cytoplasmic extract by resuspending tissue-culture

cells at a density of 2×10^9 cells/ml in HEES buffer and adjusting the suspension to concentrations of 10 mM sodium bisulfite and 1 mM dithiothreitol. The suspension was then lysed by nitrogen cavitation, as described above. The nuclei were pelleted at 15,000 × g for 2 min; the supernatant recovered is the cytoplasmic extract.

Preparation of Ribosomal Fraction

The crude cytoplasmic extract was adjusted to concentrations of 0.5 M KCl, 0.02 M sodium EDTA, 0.2% Triton X-100 and 0.2% sodium deoxycholate. The extract was then centrifuged at 20,000 × g for 10 min. The supernatant was layered on a discontinuous sucrose gradient consisting of a 5 ml column of 0.5 M sucrose over a 1 ml 2 M sucrose shelf, both prepared in HEES buffer adjusted to a concentration of 0.5 M KCl. The gradients were centrifuged at 35,000 rpm for 60 min in an SW40 rotor. The opalescent band of ribosomes floating on the shelf were recovered as the "DOC fraction." All operations were carried out at 0–4°C.

RNA Synthesis in Vitro

Nuclei (4×10^7) that had been incubated with either HEES buffer or cytoplasmic fractions at 25°C were collected by centrifugation at 1500 × g for 1 min and 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 5,6-^{3}H-uridine 5′-triphosphate or 0.03 mCi α-^{32}P-uridine 5′-triphosphate, 0.1 mg/ml pyruvate kinase (Sigma) and 12.5 mM phosphoenol pyruvate in a final volume of 0.1 ml. Reactions were carried out at 25°C for 1 hr. In experiments with E. coli RNA polymerase (purified through the high-salt Bio-Gel A-5m column step according to the method of Burgess and Jendrisak [1975]), 10 μg of the preparation were used. Fractions being assayed that were not in standard HEES buffer were adjusted to this buffer by either gel filtration with Biogel P2 or by dilution before being added to nuclei.

Purification of RNA and Hybridization Conditions

Reactions were terminated by the addition of deoxyribonuclease I (Boehringer) (30 μg/ml) for 2 min at 37°C. Samples were adjusted to concentrations of 0.5% SDS and 300 μg/ml proteinase K (Boehringer) and were incubated for 15 min at 37°C. Sodium acetate (pH 5.0) was added to a concentration of 0.2 M and the RNA was precipitated with two volumes of ethanol at −80°C.

For hybridization, the ethanol pellet was resuspended in 50 mM TES (pH 7.0), 0.6 M NaCl, 0.05 M sodium EDTA, 0.01 M sodium pyrophosphate and 60% formamide. The RNA, in a volume of 0.1 ml, was hybridized simultaneously to filters (7 mm diameter) containing approximately 2 μg of either plasmid 132E3 DNA or calf thymus DNA.

RNA probes to be used for hybridization to restriction fragments were first purified by hybridization to filters containing the entire plasmid 132E3 DNA, as described above. The RNA was recovered from the filter by incubation at 65°C for 30 min in 100% formamide and hybridized to nitrocellulose strips to which the DNA fragments had been transferred from an agarose gel according to the method of Southern (1975). This hybridization was accomplished in 60 mM TES (pH 7.0), 0.75 M NaCl, 0.05 M sodium EDTA, 16 mM sodium pyrophosphate, 0.2% SDS and 0.4 mg/ml yeast RNA in heat-sealable bags at 65°C. The filters were washed in numerous changes of 1% SDS, 1% sodium pyrophosphate in 2× SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) at 45°C. Bands were visualized by autoradiography with a Cronex intensifying screen. Radioactive 132E3 (labeled by nick translation according to the method of Rigby et al. [1977]) was hybridized to RNA immobilized to nitrocellulose either by transfer or DOT blot methods according to the method of Thomas (1980) in 60 mM TES (pH 7.0), 0.3 M NaCl, 0.25 M sodium EDTA, 10% dextran and 50% formamide at 42°C. After hybridization, filters were treated as above.

Electrophoresis

Plasmid DNA was cleaved with the appropriate restriction endonuclease in cleavage buffer (6 mM Tris–Cl [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 buffer (pH 7.0). The running buffer was also 20 mM sodium phosphate (pH 7) and the gels were run at 130 V. RNA samples were denatured according to the method of Thomas (1980) prior to electrophoresis.

SDS gel electrophoresis was carried out essentially according to the method of Laemmli (1970), and the gels were stained with Coomassie blue. Samples corresponding to about 10^6 nuclei were run in each well.

Preparation of Plasmid DNA

Plasmids 132E3 and 56H8 (provided by P. Schedl [Mirault et al., 1979]) and cDM508 (provided by D. Hogness) were prepared by precipitation of host chromosomal DNA with SDS and high salt, according to the method of 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 concentrations of 4× SSC and 10 μg/ml DNA and filtered through Schleicher and Schuell BA85 filters. The filters were baked at 80°C in a vacuum oven for 2 hr.

Acknowledgments

We gratefully express our appreciation to Dr. Paul Boyer for his help and support. This work was supported by a grant from the National Institutes of Health and by a Faculty Research Award from the American Cancer Society. B. L. C. was supported by a postdoctoral fellowship from the Damon Runyon–Walter Winchell Cancer Fund.

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Received October 21, 1980; revised May 5, 1981

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