RNA SYNTHESIS BY EXOGENOUS RNA POLYMERASE ON CYTOLOGICAL PREPARATIONS OF CHROMOSOMES

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ABSTRACT

Cytological preparations of *Drosophila* polytene chromosomes serve as templates for RNA synthesis carried out by exogenous RNA polymerase (*Escherichia coli*). Incorporation of labeled ribonucleoside triphosphates into RNA may be observed directly by autoradiography. Because of the effects of rifampicin, actinomycin D, ribonuclease, high salt, and the requirement for all four nucleoside triphosphates, we conclude that the labeling observed over chromosomes is due to DNA-dependent RNA polymerase activity. Using this method, one can observe RNA synthesis in vitro on specific chromosome regions due to the activity of exogenous RNA polymerase. We find that much of the RNA synthesis in this system occurs on DNA sequences which appear to be in a nondenatured state.

INTRODUCTION

Recent methods combining cytology with molecular hybridization (7) have strongly influenced studies of chromosome structure and function by enabling the localization of specific nucleic acid sequences. We have attempted to develop an analogous approach for the study of transcription by inducing RNA synthesis on cytological preparations of chromosomes. If cytological preparations of dipteran polytene chromosomes could be used as template for RNA synthesis, one should be able to observe transcription of specific regions directly. Such a system would permit a wide variety of treatments of the chromosomes; it would allow the addition of protein factors and polymerases, and would be a useful complement to studies of transcription in vitro with isolated nuclei, purified DNA, or isolated chromatin.

Chromosomes of various kinds fixed to slides can serve as templates for RNA synthesis. We have carried out RNA polymerase reactions on slides using fixed chromosomes and observed the incorporation of labeled ribonucleoside triphosphates by autoradiography. We have examined the requirements for RNA synthesis in this system and studied the effects of various inhibitors and treatments. RNA synthesis in this system is DNA dependent and requires exogenous RNA polymerase. New initiation of RNA chains takes place, and considerable chain elongation occurs in this system. Our results demonstrate that the chromosomal distribution of RNA synthesis in vitro can be studied at the level of cytologically defined regions of fixed polytene chromosomes.

MATERIALS AND METHODS

In general, we have adopted the methods of Gall and Pardue (8) for cytological preparations and the procedures of Burgess (5) for the purification and assay of *Escherichia coli* RNA polymerase.

Chromosome Preparations

Suspension cultures of mammalian cells were incubated overnight in colchicine at 1 μg/ml. The cells were centrifuged at low speed and swollen for 5 min in $1/4 \times$ SSC (standard saline citrate). After another low-speed spin the cells were resuspended in 3:1 ethanol-acetic acid, then centrifuged again, and gently resuspended in 45% acetic acid for 5 min before spreading or squashing on subbed slides. The slides were then frozen to remove cover slips and dehydrated with 70 and then 95% ethanol before drying in air.

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Drosophila salivary gland polytene chromosome preparations were made by dissecting glands from third instar larvae and squashing after brief treatment with 45% acetic acid (5 min). The squashes were frozen on dry ice for 15–30 min, the cover slips were removed, then slides were immediately immersed in 70% ethanol (5 min) followed by 95% ethanol for 5 min, and air dried.

To remove material from chromosomes that might affect our reaction mixtures, we treated chromosomes with HCl and RNase. HCl treatment was carried out for 30 min in 0.2 N HCl. Combined HCl-RNase treatment consisted of treating for 30 min with 0.2 N HCl, washing in 2 × SSC, treating with 100 μg/ml pancreatic RNase for 1 h at 37°C, washing again, and then treating with HCl (0.2 N, 30 min) to inactivate residual RNase. In some cases, chromosomes were treated with 0.07 N NaOH for 2 min as a denaturing treatment. After such treatments, slides were passed through ethanol and dried as above.

Enzyme Purification, Properties, and Reaction Conditions

RNA polymerase was purified from *E. coli* (K12 or B) according to the procedure of Burgess (5), using glycerol gradients to retain sigma factor. The purified enzyme was dependent on all four ribonucleoside triphosphates and was sensitive to either rifampicin, actinomycin D, or high salt (0.5 M KCl). The RNA product of the enzyme and calf thymus DNA was purified by heating, DNase, phenol extraction, and alcohol precipitation and was shown to form RNase-resistant DNA-RNA hybrids with calf thymus DNA fixed to nitrocellulose filters.

The reaction mixture follows the assay used by Burgess (5) and contain 40 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.4 mM K_3PO_4, 0.15 M KCl, and 0.5 mg/ml of bovine serum albumin. Unlabeled triphosphates were added at 0.15 mM and labeled ones were used at 0.01 mM, unless otherwise specified. Approximately 0.1 Burgess unit of enzyme were used per 100 μl of reaction mixture.

Reaction Conditions and Posttreatment for Slide Transcription

20-μl samples of reaction mixtures were placed over a chromosome preparation, covered with a glass cover slip, then incubated 1–2 h at 37°C in moist incubation dishes. After incubation, the cover slips were removed, using a 2 × SSC rinse containing 10 mM sodium pyrophosphate (pH 7.0). The slides were washed twice for 5 min each in the same solution followed by 5 min each in 70% ethanol, 95% ethanol, and then air dried. Other washes such as 0.1 × SSC or 5% TCA gave similar but not equally satisfactory results. To measure the sensitivity of the product to RNase, the slides were treated with 50 μl of pancreatic RNase at 100 μg/ml in 2 × SSC for at least 30 min after washing with 2 × SSC.

For inhibitor tests, concentrations which gave close to complete inhibition in solution were selected for use in the slide experiments. For rifampicin, 5 μg/ml was the concentration used in the assay mixture. To test the effect of actinomycin D, the chromosomes on slides were preincubated in a buffer containing 40 mM Tris-HCl pH 7.9, 0.1 mM EDTA, and actinomycin D at 25 μg/ml. After 10 min, the slides were rinsed in 40 mM Tris-HCl pH 7.9 before applying the reaction mixture. Control slides were treated identically but without inhibitor. For the high salt inhibition tests, reaction mixtures were prepared with 0.5 M KCl in place of 0.15 M KCl as in the standard reaction mixture.

Slides were dried as described, then dipped into Kodak NTB2 liquid emulsion (diluted 50% with distilled water) (Eastman Kodak Co., Rochester, N. Y.), and then exposed at 4°C in desiccated boxes for up to 33 days. After exposure, slides were placed for 2 min in D-19 developer, then 2 min in 2% acetic acid, then 2 min in Kodak fixer, followed by a 30 min rinse in distilled water. Slides were stained with Giemsa's and photographed with a Zeiss photomicroscope (Carl Zeiss, Inc., N. Y.).

DNA Minifilter RNA Polymerase Assay

Calf thymus DNA was denatured by alkali or by boiling and loaded onto Millipore nitrocellulose filters to give 100 μg of DNA per 24 mm diameter filter (Millipore Corp., Bedford, Mass.). From these filters, smaller ones (7 mm) were punched out and preincubated in 0.1 M Tris-HCl pH 7.9 and 1 mM EDTA for 30 min before use as template in a reaction mixture. Filters that were old or baked were poor template for the RNA polymerase reaction. After the reaction, the filters were treated with pancreatic RNase and vacuum washed with 2 × SSC plus pyrophosphate (0.01 M) in a miniature Millipore-type filter holder of our own construction. Filters were dried and counted in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.), using a toluene-2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene (toluene-PPO-POPOP) cocktail.

RESULTS

Immobilized DNA Can Function as Template for RNA Polymerase

As a first step in the development of a system for the study of transcription using chromosomes fixed to slides, we asked if immobilized DNA could function as template for RNA polymerase. For

this purpose, we developed an assay using denatured DNA fixed to nitrocellulose filters according to the method of Gillespie and Spiegelman (9). If such DNA filters did work as templates for RNA polymerase, we expected that transcription on slides would also be possible. RNA synthesis was observed as labeled nucleotide incorporated from a standard reaction mixture into filter-bound RNase-resistant radioactivity. In Fig. 1, we compare the incorporation of label into filter-bound material, when all components are present (*a*), when one triphosphate is left out (*b*), and when DNA is not present on the filters (*c*). The incorporation appears to be due to the formation of DNA-RNA hybrids by activity of DNA-dependent RNA polymerase. We conclude that *E. coli* RNA polymerase is active on immobilized, denatured

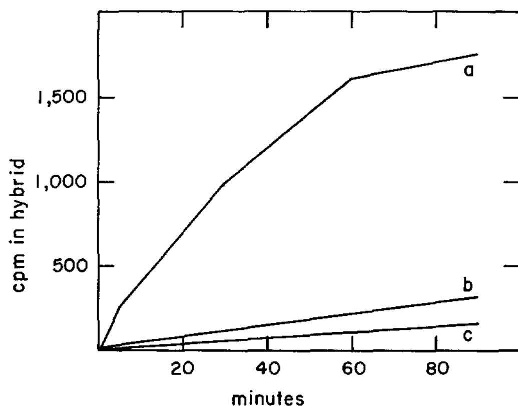


FIGURE 1 The DNA-dependent synthesis of RNA on minifilters is shown as an increase in labeled nucleotide incorporated into RNase-resistant DNA-RNA hybrid. Curve *a* shows the time-course of labeling using a complete reaction mixture and DNA filters. Curve *b* shows a similar reaction which lacked one of the unlabeled triphosphates (ATP). Curve *c* shows results of a complete reaction mixture, but using filters without DNA. The labeled nucleotide used was GTP at 0.2 Ci/mmol. Enzyme (Burgess GG2 enzyme) was added at 0.02 U/100 μl. In control experiments with added DNA in solution (undenatured) under similar conditions, a complete reaction gave 14,438 cpm incorporated as TCA-precipitable counts per minute. Leaving out ATP gave 337 cpm; without DNA but including ATP gave 173 cpm, and no ATP and no DNA gave 196 cpm. The DNA concentration was low, 8 μg, because an attempt was made to use the same amount as ordinarily used on the minifilter. Time points for all curves include 5, 30, 60, and 90 min. Each minifilter was incubated in a reaction volume of 50 μl.

DNA. We therefore directed our attention to the synthesis of RNA on chromosomes after they had been fixed to glass slides and subjected to denaturing treatments.

Incorporation of Labeled Ribonucleotides Using Cytological Preparations of Chromosomes as Template

Chromosome squashes were prepared as described in Materials and Methods. Denaturing treatments were applied because, due to our minifilter results, we inferred that RNA synthesis would be detectable as enzymatic DNA-RNA hybrid, but that synthesis of RNA from native template would be more difficult to detect. Up to 50 μl of various reaction mixtures were applied to slides of Chinese hamster metaphase chromosomes, and the slides were incubated 30 min at 37°C, washed in turn with 2 $\times$ SSC, 70% ethanol, 95% ethanol, and dried. To determine label incorporated, the slides were fragmented and counted directly in toluene-PPO-POPOP scintillation fluid. The results of such tests are shown in Table I. Labeled ribonucleotides appear to be incorporated with a two- to tenfold increase between treated slides and controls. While the amount of label incorporated was low, the results suggest that slides prepared in the manner described act as template for exogenous RNA polymerase. To show that the label on the slides was in fact associated with the chromosomes, similarly treated slides were prepared using HeLa cells, incubated as above with reaction mixtures, washed, dried, and prepared for autoradiography. Extensive labeling was found over the chromosomes as shown in Fig. 2 *a*. This labeling observed is greatly decreased in the absence of the enzyme (Fig. 2 *c*) or in the presence of rifampicin (Fig. 2 *b*). Similar labeling has been observed using Chinese hamster chromosomes and chromosomes from neural ganglia of *Drosophila* third instar larvae.

RNA Synthesis on Drosophila Polytene Chromosomes

Since the value of this approach depends upon the ability to resolve RNA synthesis of specific chromosome regions, we have carried out our most extensive tests on *Drosophila* salivary gland chromosomes. The puffing patterns of the banded chromosomes of *Drosophila melanogaster* have been extensively described (2, 3). The use of polytene

TABLE I
Incorporation of Labeled Precursor on Slides Treated with RNA Polymerase Reaction Mixtures

Exp.	Pretreatment	Incubation	Posttreatment	cpm	
1	HCl, RNase, NaOH	Complete	2 × SSC, 70 and 95% EtOH	1,286	1,063
1	HCl, RNase, NaOH	No enzyme	2 × SSC, 70 and 95% EtOH	191	187
1	HCl, RNase	Complete	2 × SSC, 70 and 95% EtOH	1,005	849
1	HCl, RNase	No enzyme	2 × SSC, 70 and 95% EtOH	133	130
2	HCl, RNase, NaOH	Complete	5% TCA, 70 and 95% EtOH	624	475
2	HCl, RNase, NaOH	No enzyme	5% TCA, 70 and 95% EtOH	126	116
2	HCl, RNase, NaOH	Complete	2 × SSC, 70 and 95% EtOH	345	174
2	HCl, RNase, NaOH	No enzyme	2 × SSC, 70 and 95% EtOH	46	39
3	HCl, RNase	Complete	2 × SSC, 70 and 95% EtOH	238	214
3	HCl, RNase	Minus CTP, UTP, ATP	2 × SSC, 70 and 95% EtOH	129	119
4	HCl, RNase, NaOH	Complete	5% TCA, 70 and 95% EtOH	481	375
4	HCl, RNase, NaOH	No enzyme	5% TCA, 70 and 95% EtOH	144	92
4	HCl, RNase, NaOH	Complete	2 × SSC, 70 and 95% EtOH	669	449
4	HCl, RNase, NaOH	No enzyme	2 × SSC, 70 and 95% EtOH	306	213

Chromosome squashes were prepared from colchicine-arrested Chinese hamster cells as described in Materials and Methods. In the Table, pretreatment refers to the procedures used to prepare slides for the reactions after fixing and squashing cells. Incubation describes the conditions of reaction mixture during incubation for 30 min at 37°C. After incubation the slides were washed as described in pretreatment for 5 min for each type of wash. Counts per minute are given as total counts per minute after washing, from each slide fragmented and counted in a toluene-PPO-POPOP scintillation fluid. The reaction conditions were basically those of Burgess as modified in Materials and Methods. The labeled nucleoside triphosphate in these reactions was [^{3}H]GTP. Each pair of numbers in the last two columns represents duplicate slides, fragmented and counted.
EtOH, ethanol.

chromosomes enables an eventual comparison of the distribution of transcribed sites, using exogenous polymerase, with the in vivo distribution of active sites. Consequently, we prepared chromosome squashes from *Drosophila* salivary glands and carried out similar reactions as described. Fig. 3 presents a spread of a *Drosophila* salivary gland nucleus showing labeling of most regions of the chromosomes. The nucleolus, however, shows very little labeling. In order to verify that we were in fact observing RNA synthesis and to learn more about the system, we have examined the relative effectiveness of a variety of treatments, reaction conditions, and inhibitors. A summary of these results is shown in Table II. Some specific groups of experiments are discussed in the following sections.

Effects of Rifampicin and High Salt

Rifampicin and high salt (0.5 M KCl) are strong inhibitors of the initiation of transcription by *E. coli* RNA polymerase (13). In order to determine that initiation of RNA synthesis was in fact taking place, we examined the effect of these conditions on the labeling of salivary gland chromosomes. Fig. 4 *a* shows an uninhibited complete reaction mixture; Fig. 4 *b* shows the effect of rifampicin; Fig. 4 *c* shows the effect of 0.5 M KCl. Both rifampicin and high salt strongly inhibit the extent of labeling. Inhibition appears to occur for all regions of the chromosomes. The results indicate that initiation of transcription is required for the labeling we observe. However, it remained to demonstrate that chain elongation was taking place in addition to the binding of the first nucleotide.

Evidence for Chain Elongation

In order to show that chain propagation occurs after the formation of initiation complexes, we

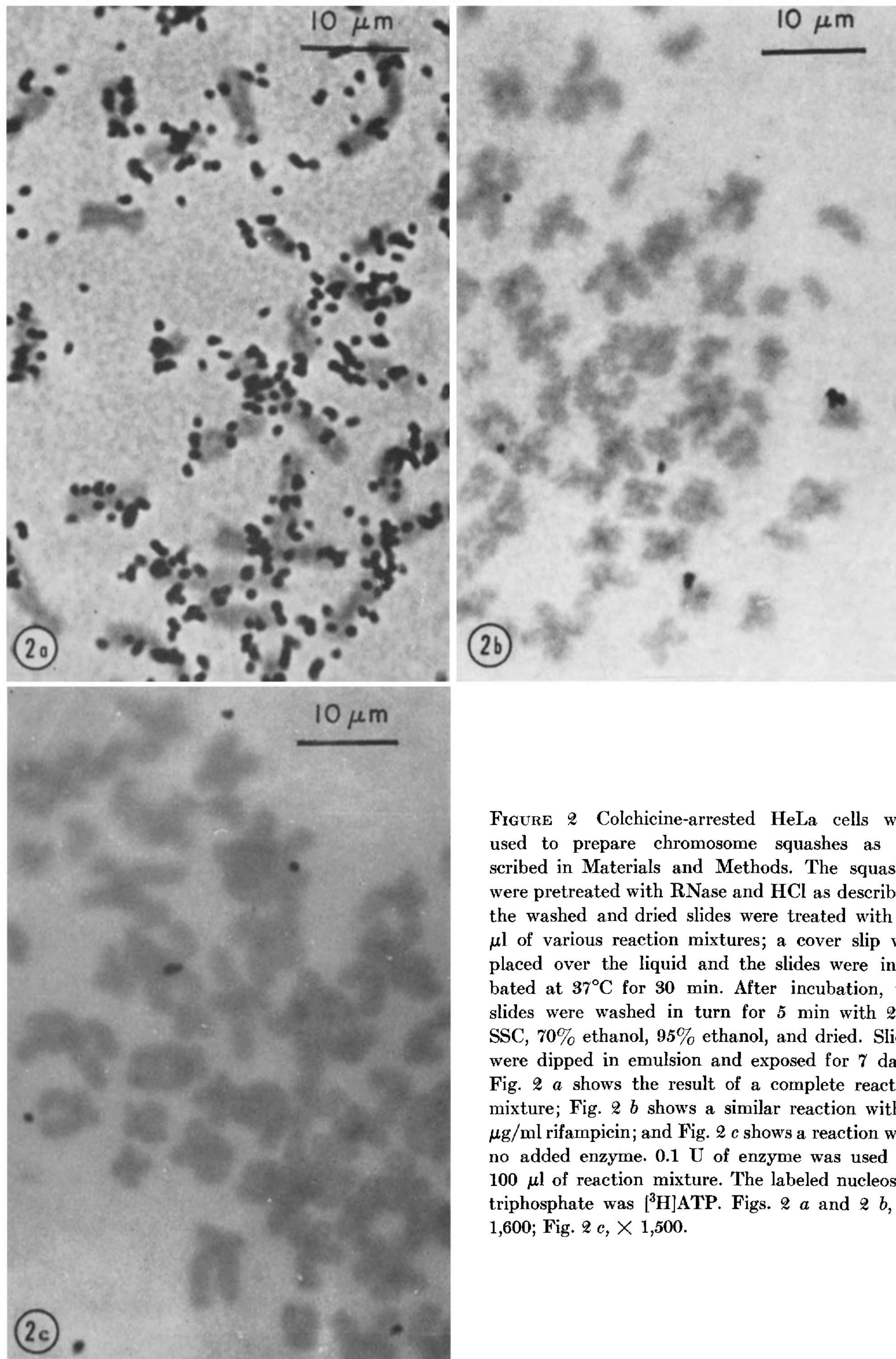


FIGURE 2 Colchicine-arrested HeLa cells were used to prepare chromosome squashes as described in Materials and Methods. The squashes were pretreated with RNase and HCl as described; the washed and dried slides were treated with 20 μl of various reaction mixtures; a cover slip was placed over the liquid and the slides were incubated at 37°C for 30 min. After incubation, the slides were washed in turn for 5 min with 2 × SSC, 70% ethanol, 95% ethanol, and dried. Slides were dipped in emulsion and exposed for 7 days. Fig. 2 *a* shows the result of a complete reaction mixture; Fig. 2 *b* shows a similar reaction with 5 μg/ml rifampicin; and Fig. 2 *c* shows a reaction with no added enzyme. 0.1 U of enzyme was used for 100 μl of reaction mixture. The labeled nucleoside triphosphate was [^{3}H]ATP. Figs. 2 *a* and 2 *b*, × 1,600; Fig. 2 *c*, × 1,500.

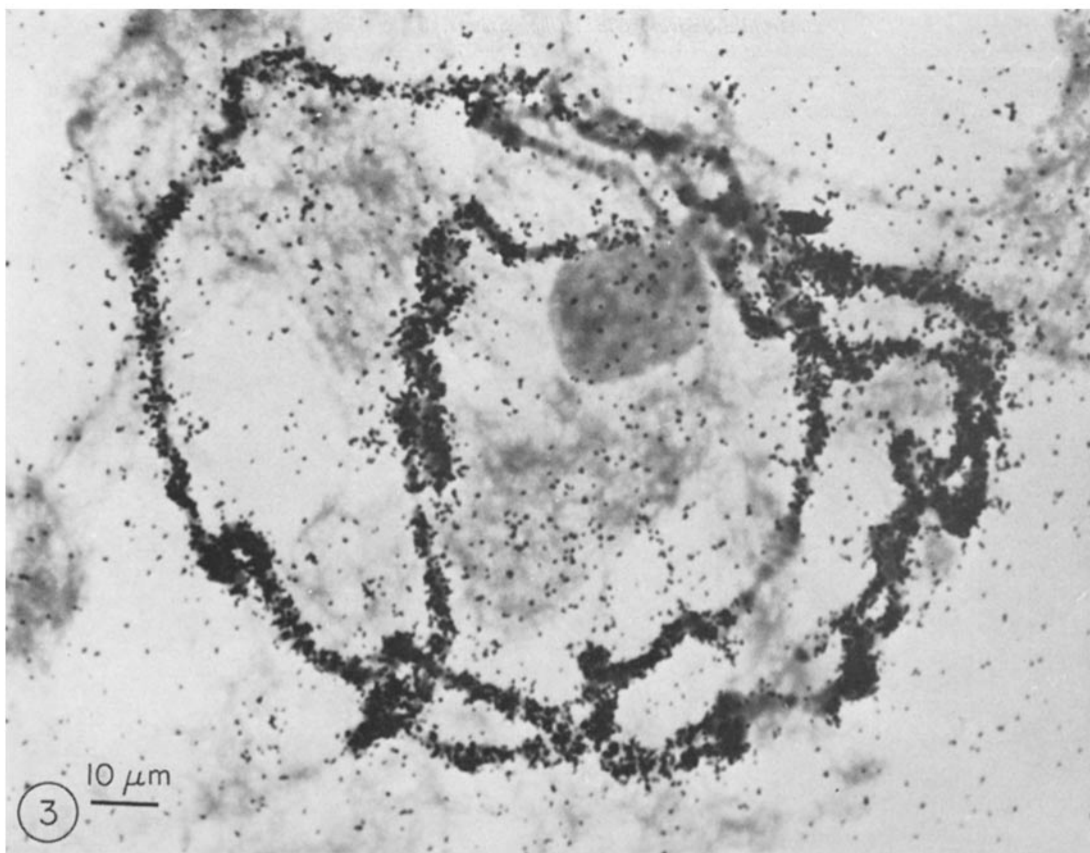


FIGURE 3 Salivary glands from *D. hydei* were dissected in 45% acetic acid, squashed, frozen on dry ice, treated with HCl, washed with 70 and then 95% ethanol, and dried before reaction mix was applied. The slides were incubated with a complete reaction mix containing ATP and GTP as labeled triphosphates. After the reaction, the slides were washed in turn with 2 × SSC, 70% ethanol, and 95% ethanol. Exposure time was 19 days. Photograph was taken from Experiment 6 in Table II. × 900.

asked if the labeling reaction depended on the presence of all four nucleoside triphosphates. Labeling is greatly reduced in the absence of one unlabeled nucleoside triphosphate and completely inhibited by the absence of two unlabeled nucleoside triphosphates. These results are shown in Fig. 5 and Table II. The observed dependence on all four triphosphates is strong evidence that RNA synthesis is taking place on chromosome preparations rather than purely initiation complexes or certain nonspecific artifacts.

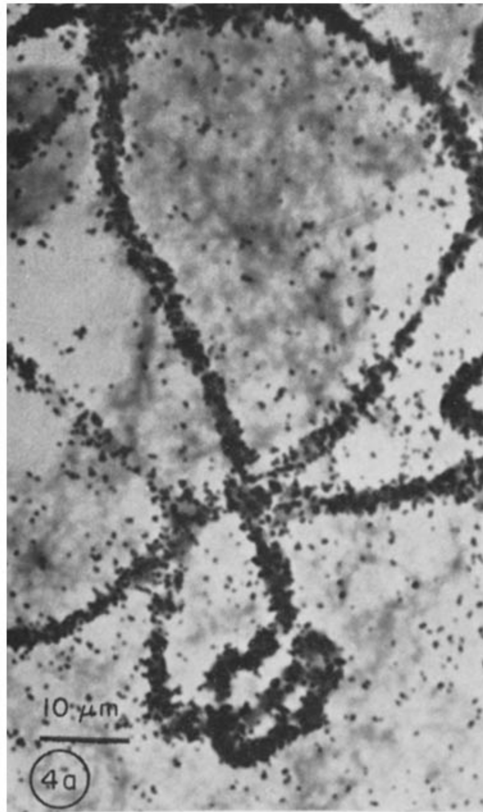
Further support for chain elongation comes from experiments with actinomycin D, which is known to bind to DNA and to inhibit RNA synthesis during propagation. Before the application of a reaction mixture, chromosomes on slides were treated with actinomycin D and then washed with Tris-HCl pH 7.9. Controls were treated identically but without addition of actinomycin. After washing, all slides were treated with a standard complete reaction mixture. The results in Fig. 6 show that actinomycin D strongly inhibits the labeling reaction. Tests for actinomycin D inhibition have been carried out using chromosomes treated or not treated with HCl. In both cases, labeling is inhibited if the chromosomes are pretreated with actinomycin D. We conclude that chain elongation does occur in this system. As was the case with rifampicin and high salt, all chromosome regions seem to be equally inhibited.

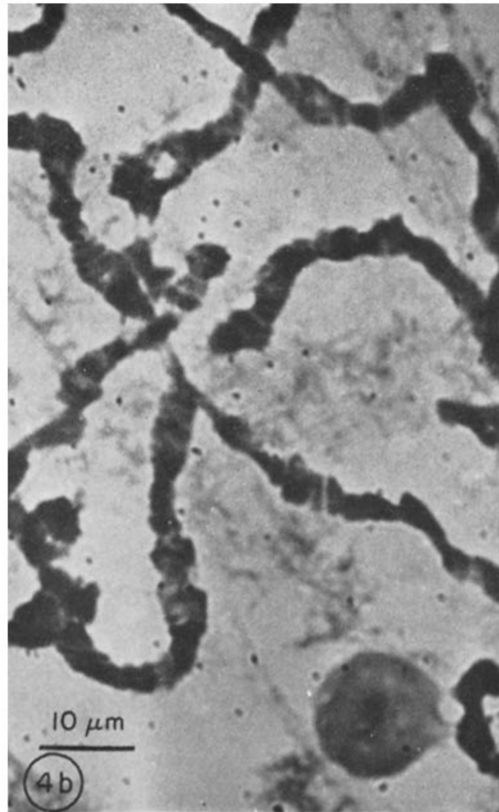
TABLE II

In Situ Transcription of Drosophila Salivary Gland Chromosomes, Using Various Conditions for Reactions, Chromosome Preparations, and Washing

Exp.	No. of slides	Pretreatment	Incubation mix	Reaction time	Posttreatment	Exposure days	Labeling on chromosomes
				min			
1	7	HCl	Complete	60	0.1 × SSC, 70 and 95% EtOH	33	Heavy
1	3	HCl	Minus UTP	60	0.1 × SSC, 70 and 95% EtOH	33	Negative
1	3	HCl	Minus CTP and UTP	60	0.1 × SSC, 70 and 95% EtOH	33	Negative
1	3	HCl	Minus CTP, UTP, ATP	60	0.1 × SSC, 70 and 95%, EtOH	33	Negative
2	5	HCl	Complete	120	RNase, 2 × SSC, 70 and 95%, EtOH	25	Negative
2	5	HCl	Complete	120	2 × SSC, 70 and 95% EtOH	25	Light
2	5	HCl	Complete	120	70% and 95% EtOH	25	Moderate
2	5	HCl	Complete	120	95% EtOH	25	Heavy
2	5	HCl	Complete	120	0.1 × SSC	25	Heavy
3	8	HCl	Complete	60	2 × SSC, 70 and 95% EtOH	21	Heavy
3	4	HCl	Minus one triphosphate	60	2 × SSC, 70 and 95% EtOH	21	Light
3	4	HCl	Minus CTP and UTP	60	2 × SSC, 70 and 95% EtOH	21	Negative
4	5	HCl	Complete	60	2 × SSC, 70 and 95% EtOH	21	Heavy
4	5	HCl	Complete	60	0.1 × SSC, 70 and 95% EtOH	21	Heavy
5	8	HCl	Complete	60	0.1 × SSC, 70 and 95% EtOH	20	Heavy
5	3	HCl	Minus UTP	60	0.1 × SSC, 70 and 95% EtOH	20	Light
5	4	HCl	Minus CTP and UTP	60	0.1 × SSC, 70 and 95% EtOH	20	Negative
6	8	HCl	Complete	60	2 × SSC, 70 and 95% EtOH	19	Heavy
6	4	HCl	Complete + rif	60	2 × SSC, 70 and 95% EtOH	19	Negative
6	4	HCl	Complete in 0.5 M KCl	60	2 × SSC, 70 and 95% EtOH	19	Negative-light
7	9	HCl	Complete	60	2 × SSC (30 min), 70 and 95% EtOH	14	Heavy-moderate
7	9	HCl	Complete	60	RNase (30 min), 70 and 95% EtOH	14	Light
8	8	HCl	Complete	60	2 X SSC, 70 and 95% EtOH	12	Heavy
8	8	HCl	Complete	60	5% TCA, 70 and 95% EtOH	12	Heavy
8	8	None	Complete	60	2 × SSC, 70 and 95% EtOH	12	Heavy
9	10	HCl	Complete	60	2 × SSC, 70 and 95% EtOH	9	Moderate
9	5	None	Complete	60	2 × SSC, 70 and 95% EtOH	9	Moderate
9	5	HCl	Complete + act D	60	2 × SSC, 70 and 95% EtOH	9	Negative
9	5	None	Complete + act D	60	2 × SSC, 70 and 95% EtOH	9	Negative

A series of transcription experiments are shown with various treatments of chromosomes before the reaction (pretreatments), using different reaction mixtures during incubation at 37°C (incubation mix), and with various washing treatments after the reactions (posttreatment). When an incubation mixture is listed as minus CTP, the unlabeled CTP has been left out of the reaction mix. Those triphosphates listed as such are not labeled in those reaction mixtures. For example, in Experiment 1 the labeled triphosphate in all sets was GTP. No more than two labeled triphosphates were used in any single experiment. Reaction time is given in minutes at 37°C, unless specified. All other steps were carried out at room temperature. Experiments 1–8 used chromosomes from *D. hydei* while Experiment 9 used chromosomes from *D. melanogaster*. The meaning of the designations for degree of labeling may be determined by examining photogaphs shown, taken from these experiments, i.e., Experiments 3, 6, 7, or 9.





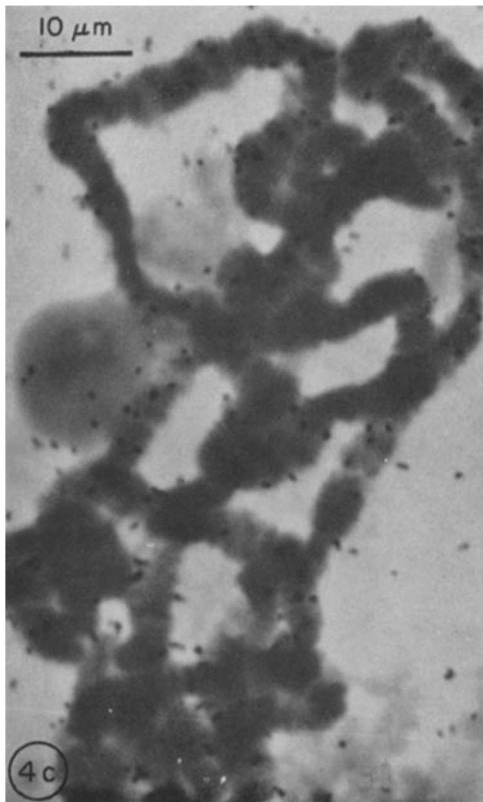


FIGURE 4 Photographs taken from Experiment 6 in Table II. Fig. 4 *a* shows a portion from a heavily labeled chromosome squash from a complete reaction; Fig. 4 *b* shows a parallel squash treated in the same way but in the presence of 5 μg/ml rifampicin; Fig. 4 *c* shows a parallel sample treated with a reaction in 0.5 M KCl. Fig. 4 *a*, × 1,200; Fig. 4 *b*, × 1,250; Fig. 4 *c*, × 1,500.

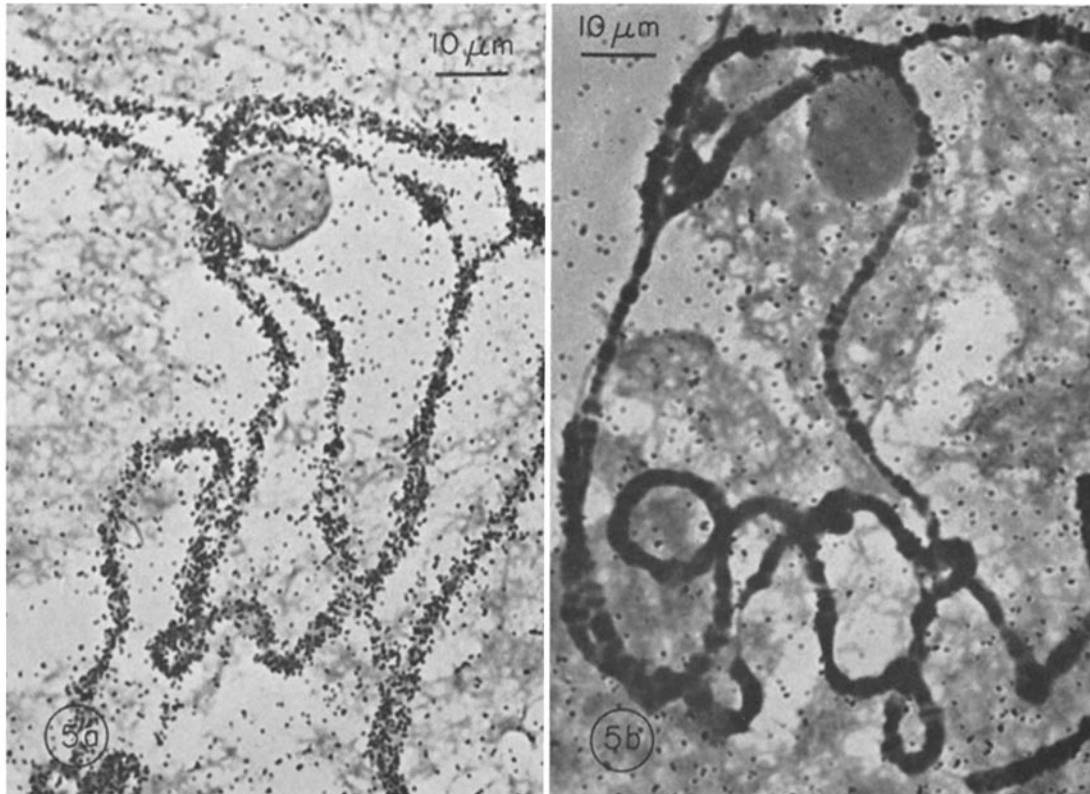


FIGURE 5 Fig. 5 *a* shows a part of a chromosome squash from Experiment 3 (in Table II) showing labeling from a complete reaction. Fig. 5 *b* shows the extent of labeling when unlabeled CTP and UTP are left out. Both ATP and GTP are present as labeled bases at undiluted specific activity. Fig. 5 *a*, × 950; Fig. 5 *b* × 1,000.

Effect of RNase

To verify that the product of the labeling reaction was RNA, we treated slides with pancreatic RNase. Slides treated with a complete reaction mixture were divided into two groups, one was exposed to pancreatic RNase at 100 µg/ml in 2 × SSC, and the other was treated with 2 × SSC alone. The results are shown in Fig. 7. Most if not all of the label was removed by RNase. All regions of the chromosomes were equally affected. If the DNA in the chromosome preparations was in a denatured state, as we originally expected, we would expect the labeled material to be resistant to RNase. Under our conditions, the RNA product made in solution on heat-denatured or acid-denatured DNA is largely resistant to RNase whereas the product made from native DNA in solution is largely sensitive. Assuming that the specificity of RNase activity on cytological preparations is the same as it is in solution, we infer that a large fraction of DNA in our preparations is in a nondenatured state during the reaction.

The inference that the major fraction of the DNA is in a renatured state is supported by the results in the previous section showing inhibition of the reaction by actinomycin D, since actinomycin is known to bind strongly to native but not denatured DNA (14). Because all regions appear equally affected by RNase, it appears that there are not large localized regions of single strandedness in these chromosome preparations.

Distribution of Labeling

Fig. 8 *a* shows a labeled preparation from *D. melanogaster* of the tip of the X chromosome. Fig. 8 *b* shows the tip of the X in a temporary preparation for comparison of the banding pattern. Labeling seems to be heaviest in banded regions and

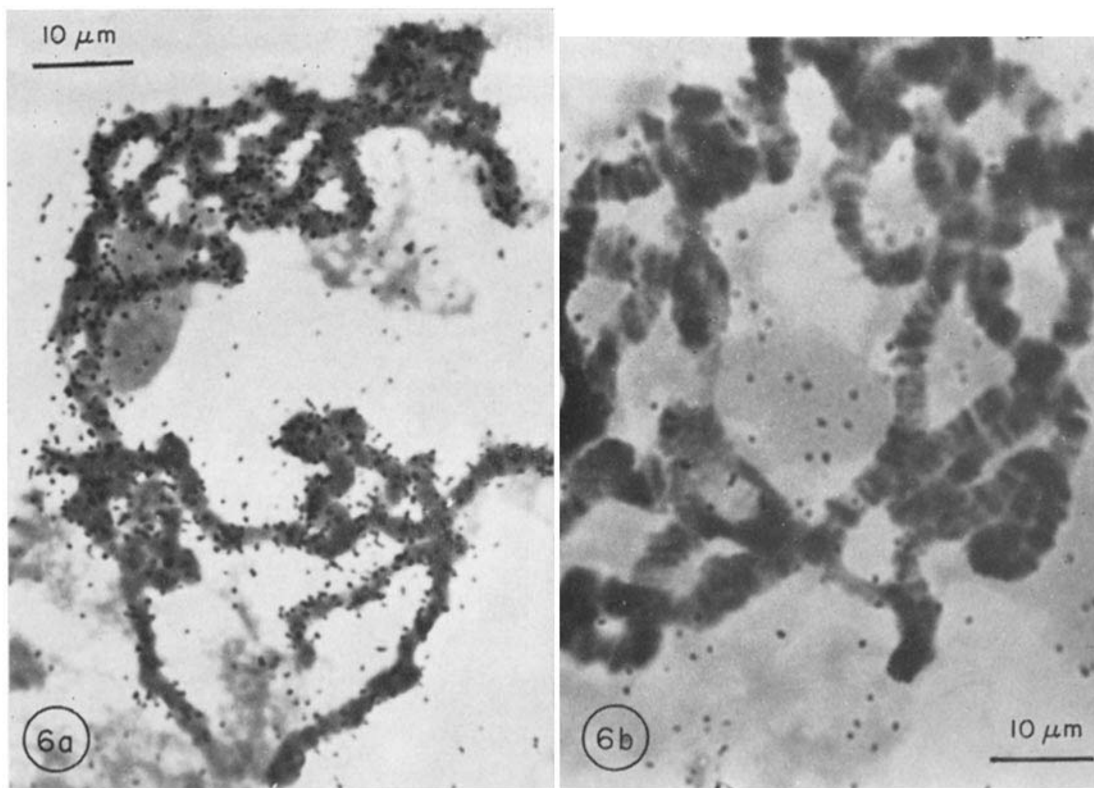


FIGURE 6 Photographs were taken of slides from Experiment 9 of Table II. Fig. 6 *a* shows a result of a complete reaction mixture on slides preincubated in 25 μg/ml of actinomycin D in 0.04 M Tris-HCl pH 7.9 and 0.1 mM EDTA. Fig. 6 *b* shows a similar set preincubated with the same buffer but without actinomycin D. × 1,400.

light for puffed regions. We suggest that the increased labeling is due either to the higher DNA content of the bands or to a differential distribution of other factors which affect our labeling reaction. The same possibilities apply for an explanation of the low level of labeling of the nucleolus. Further studies may enable us to understand the basis of the labeling distribution.

DISCUSSION

Other workers have previously used enzymes that work on nucleic acids as probes for investigating chromosome structure. Von Borstel and co-workers (15, 16) have used DNA polymerase and terminal transferase on a variety of cytological preparations of chromosomes. DNA polymerase appeared to be a useful tool for the detection of single-stranded regions in chromosomes. Terminal transferase was able to detect the presence of ends of DNA chains. Since DNA polymerase does not function on native DNA, activity was observed after treatments which denatured DNA. In such cases, it is difficult to determine what fraction of the DNA is in a native or denatured state.

Endogenous RNA polymerase activity in cytological preparations has been measured previously using autoradiography. Fisher (6) measured endogenous RNA polymerase activity in frozen sections of plant tissues. Moore (10) has measured endogenous RNA polymerase activity in methanol-fixed cells during spermatogenesis. Berendes and Boyd (4) and recently Alonso (1) have examined endogenous RNA synthesis in isolated *Drosophila* nuclei containing polytene chromosomes.

We have focused our attention on the development of a system for studying transcription using chromosomes fixed on slides because of the potential for observation of site-specific RNA synthesis under conditions where exogenous substrates, enzyme, and protein factors could be readily applied.

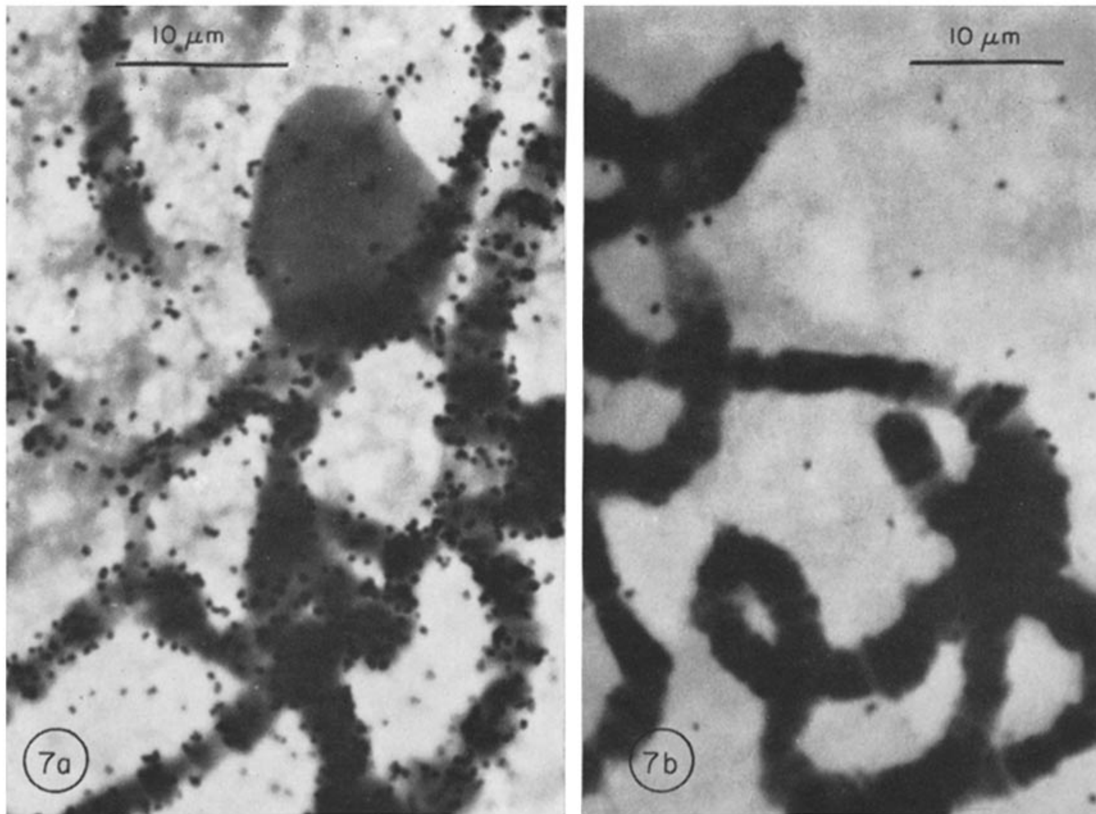


FIGURE 7 Salivary gland chromosomes from *D. melanogaster* were fixed and squashed in 45% acetic acid, HCl treated, and prepared for a standard reaction mix. Labeled ATP and GTP were used. Incubation was 1 h. After incubation, the cover slips were removed and slides in two groups were treated either with RNase (Fig. 7 *b*) at 100 µg/ml in 2 × SSC or with 2 × SSC alone (Fig. 7 *a*). After 30 min at 37°C, all slides were washed with 2 × SSC plus pyrophosphate and then with 70 and 95% ethanol. Slides were then prepared for autoradiography, and exposed for 14 days. Photographs shown are from Experiment 7, Table II. Fig. 7 *a*, × 2,300; Fig. 7 *b*, × 2,000.

We expect that a comparison of patterns of synthesis in systems such as we present here with those in systems using intact nuclei, will yield information about the factors which determine site-specific RNA synthesis.

In the experiments reported here, the chromosome preparations have been treated with acetic acid and, often, also with HCl. Two lines of evidence indicate that these treatments produce denatured regions in these chromosomes. Activity of DNA polymerase on such types of chromosome preparations (15) indicates single-stranded regions, because DNA polymerase requires a single-stranded template. Moreover, Gall and co-workers (7, 8) prepare chromosomes in a similar way for cytological hybridizations. Their procedures must produce some denatured material which permits the formation of DNA-RNA hybrids. Both cytological hybridization and activity of DNA polymerase do not detect sequences which might not be denatured due to some restriction, or which renature very rapidly due to the proximity of the complementary strands. Our evidence on RNase sensitivity and inhibition by actinomycin D supports the interpretation that a large fraction of the chromosomal DNA is in a nondenatured state in our cytological preparations.

This interpretation has been advanced previously by Nash and Plaut (11) who monitored the native or denatured state of the chromosomes using the fluorochrome acridine orange. They demonstrated that the native or renatured state was

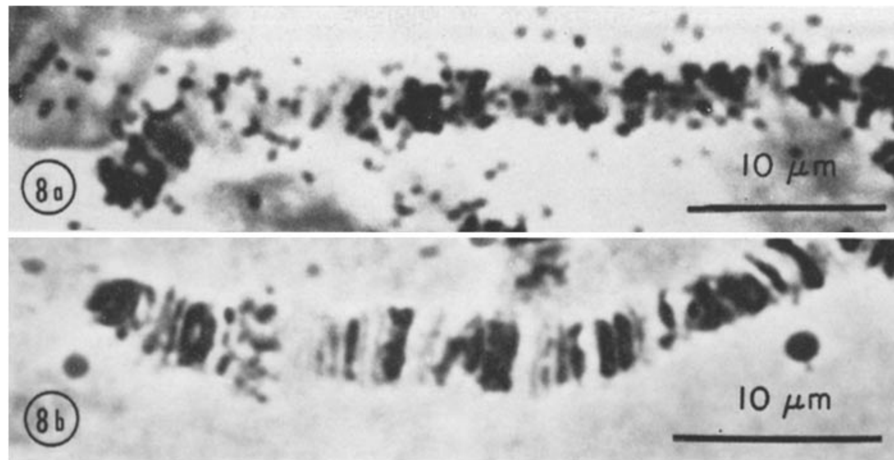


FIGURE 8 The tip of the X chromosome of *D. melanogaster* is shown in Fig. 8 *a* after labeling with a complete reaction mixture followed by autoradiography for 38 days. ATP was the labeled triphosphate. Slides were washed with 2 × SSC, 70% ethanol, 95% ethanol, and air dried before autoradiography. The chromosomes were prepared by acetic acid squashes without further acid treatment. Fig. 8 *b* shows a squash fixed with acetic acid, stained with lactoacetic orcein, and photographed under a phase-contrast microscope. Fig. 8 *a*, × 2,600; Fig. 8 *b*, × 2,800.

found after acid treatments similar to those used in our experiments. In our laboratory, J. Kaunitz has examined the fluorescence of acridine orange with our chromosome preparations and obtained similar results. A similar conclusion may be drawn from the work of Plessmann Camargo and Plaut (12). They demonstrated that actinomycin D binds to acid-treated salivary gland chromosomes, (actinomycin D is known to bind specifically to double-stranded DNA [14]). It would seem reasonable to conclude that chromosomal preparations can be made which have most of the DNA in a nondenatured state and that such chromosomes can function as templates for transcription by exogenous RNA polymerase *in situ*.

It should be mentioned that our treatments cause the removal of basic proteins and some other soluble components from the chromosomes. We are particularly interested in examining unfixed chromosomes in vitro to investigate the role of basic proteins in the distribution of transcriptionally active regions. Our approach to in vitro studies of transcriptional specificity offers advantages over conventional methods using purified DNA or isolated chromatin. In our system the chromosomal distribution of transcription products may be determined directly, independent of renaturation rates or mispairing problems found in molecular hybridization analysis. We conclude that all major regions of the salivary chromosomes, except for the nucleolus, appear to be equally accessible to *E. coli* RNA polymerase and are apparently equally transcribed in these types of chromosome preparations.

The value of this technique for future studies of RNA synthesis depends on comparisons of labeling patterns in vitro and in vivo using chromosomes subjected to various treatments and using polymerases and chromosomal proteins from *Drosophila*. Because permanent lines of embryonic *Drosophila* cells are now in culture, such protein fractions from a uniform cell type are becoming available. We hope to examine such enzymes and chromosomal proteins in our system, and since our preparations show relatively unrestricted transcription, we can look for factors or enzymes which carry out restricted synthesis of specific chromosomal regions.

This research was supported by grants from the National Science Foundation (GB 21225) and the National Institutes of Health (1-R01-GM-18079-01).

Received for publication 19 September 1972, and in revised form 20 November 1972.

Note Added in Proof: We wish to acknowledge the work of Umiel and Plaut which appeared while this article was in press. Some of their experiments were

similar to ours and they obtained similar results. See Umiel, N., and W. Plaut. 1973. *J. Cell Biol.* **56**:139.

REFERENCES

1. Alonso, C. 1972. The influence of molting hormone on RNA synthesis in isolated polytene nuclei of *Drosophila. Dev. Biol.* **28**:372.
2. Ashburner, M. 1967. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. I. Autosomal puffing patterns in a laboratory stock of *Drosophila melanogaster. Chromosoma.* **21**:398.
3. Ashburner, M. 1969. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. II. The X chromosome puffing patterns of *D. melanogaster* and *D. simulans. Chromosoma.* **27**:47.
4. Berendes, H. D., and J. B. Boyd. 1969. Structure and functional properties of polytene nuclei isolated from salivary glands of *Drosophila hydei. J. Cell Biol.* **41**:591.
5. Burgess, R. R. 1969. A new method for the large scale purification of *Escherichia coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase. *J. Biol. Chem.* **244**:6160.
6. Fisher, D. B. 1968. Localization of endogenous RNA polymerase activity in frozen sections of plant tissues. *J. Cell Biol.* **39**:745.
7. Gall, J. G., and M. L. Pardue. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. U. S. A.* **63**:378.
8. Gall, J. G., and M. L. Pardue. 1971. Nucleic acid hybridizations in cytological preparations. *Methods Enzymol.* **21D**:470.
9. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybridization with DNA immobilized on a membrane. *J. Mol. Biol.* **12**:829.
10. Moore, G. P. M. 1971. DNA dependent RNA synthesis in fixed cells during spermatogenesis in mouse. *Exp. Cell Res.* **68**:462.
11. Nash, D., and W. Plaut. 1964. On the denaturation of chromosomal DNA *in situ. Proc. Natl. Acad. Sci. U. S. A.* **51**:731.
12. Plessmann Camargo, E., and W. Plaut. 1967. The radioautographic detection of DNA with tritiated actinomycin D. *J. Cell Biol.* **35**:713.
13. Richardson, J. P. 1969. RNA polymerase and the control of RNA synthesis. *Prog. Nucleic Acid Res. Mol. Biol.* **9**:75.
14. Sobell, H. M., and S. C. Jain. 1972. Stereochemistry of actinomycin binding to DNA. II. Detailed model of actinomycin-DNA complex and its implications. *J. Mol. Biol.* **68**:21.
15. Von Borstel, R. C., O. L. Miller, Jr., and F. J. Bollum. 1969. Probing the structure of chromosomes with DNA polymerase and terminal transferase. *Genetics.* **61**(Suppl.):401.
16. Von Borstel, R. C., D. M. Prescott, and F. J. Bollum. 1966. Incorporation of nucleotides into nuclei of fixed cells by DNA polymerase. *J. Cell Biol.* **29**:21.