Association of a Protease with Polytene Chromosomes of

Drosophila melanogaster

JOY CAVAGNARO, DOROTHY A. PIERCE, JOHN C. LUCCHESI, and CHI-BOM CHAE
Departments of Biochemistry and Zoology, University of North Carolina, Chapel Hill, North Carolina 27514

ABSTRACT   Incubation of *Drosophila* salivary glands with radioactive diisopropyl fluorophosphate results in the uniform labeling of polytene chromosomes. Extensive labeling is seen only when chromosome squashes are prepared by a formaldehyde fixation procedure and not by standard acetic acid techniques. The labeling is inhibited in the presence of tosylphenylalanine chloromethyl ketone and phenylmethane sulfonylfluoride but not by tosyllysine chloromethyl ketone, suggesting that a chymotrypsin-like serine protease is associated with the chromosomes. Protease inhibitors show no apparent effect on heat-shock specific puffing.

Chromatin isolated from various rat and mouse tissues contains a highly basic 25,000-dalton serine protease (3), which appears to be directly bound to the DNA (J. Cavagnaro and C.-B. Chae, manuscript submitted for publication). The protease selectively cleaves H1 histone in condensed but not in relaxed chromatin (11, 12) and oligonucleosomes in vitro (Y.-J. Kim and C.-B. Chae, unpublished results), suggesting the possibility of involvement of the enzyme in the local decondensation of super-coiled chromatin fiber by controlled cleavage of H1 histone in vivo. The polytene chromosomes of *Drosophila melanogaster* larval salivary glands are an excellent system with which to study this possibility because specific genes at certain loci can be activated by heat and this gene activation accompanies readily recognizable puffing at the heat-shock specific loci (18). Therefore, we have attempted to establish that a protease is associated with polytene chromosomes by labeling chromosomes with a radioactive active site-directed protease inhibitor, diisopropyl fluorophosphate (DFP) and have studied the possible effect of protease inhibitors on heat-shock specific puffing.

We find that although a chymotrypsin-like protease appears to be uniformly distributed along the polytene chromsomes, protease inhibitors do not appear to affect the specific puffing elicited by heat shock.

MATERIALS AND METHODS

Labeling of Salivary Glands with [³H]DFP

Salivary glands from third instar larvae of *D. melanogaster* were dissected into insect Ringer's solution (8). After dissection, two sets of glands were placed on a welled slide containing 50 μl of *Drosophila* phosphate-buffered saline solution (1) plus protease inhibitor or solvent used for inhibitor. Slides were sealed with a ring of silicone grease and a coverslip and incubated at room temperature for 30 min. The glands were then transferred to 50 μl of *Drosophila* phosphate-buffered solution (PBS) containing 6.15×10^{-5} M [³H]DFP and incubated for 30 min. After incubation the glands were transferred to formaldehyde fixative for 1-2 min and then squashed in formaldehyde-acetic acid (22) onto a subbed slide. The slide was then dipped into liquid nitrogen. Immediately after dipping, the coverslip was removed with a razor blade and the slide was placed in cold PBS for 30-120 min with three changes of buffer. After the last rinse in PBS, the slides were prepared for autoradiography. Autoradiography and Giemsa staining were performed as previously described (15).

Heat Shock

For heat-shock experiments the salivary glands were incubated at 37°C for 30 min in *Drosophila* PBS.

SDS Gel Electrophoresis of [³H]DFP-labeled Proteins

20 salivary glands were incubated in 100 μl of *Drosophila* phosphate-buffered solution with or without inhibitor and incubated for 30 min at room temperature. The solution was removed with a tuberculin syringe and 100 μl of [³H]DFP in PBS (final concentration 6.15×10^{-5} M [³H]DFP) was applied to cover the glands. After 30-min incubation, the label was removed with a syringe and 100 μl of formaldehyde fixative solution (22) was added. After a 20-min incubation, 20 μl of cold 20% TCA was added and the glands were then transferred to 3 ml of the same solution and incubated for 10 min at 0°C. Protein was extracted from the fixed and acid-precipitated glands according to the method of Tissieres et al. (24) and electrophoresed in 9.5% SDS polyacrylamide gels according to Laemmli (14). The radioactivity of the gel slices was determined as previously described (3).

Assay of Protease Activity of Drosophila Embryo Chromatin

Pure nuclei were prepared from 6- to 18-h *Drosophila* embryos by centrifugation through 2.2 M sucrose, 0.01 M Tris-maleate (pH 7.4), 5 mM MgCl₂ (7), and chromatin was prepared by repeated washing of the nuclei with 0.075 M

NaCl–0.024 M EDTA (pH 7.9), 50 mM Tris, pH 7.9, and 5 mM Tris, pH 7.9. An aliquot was sonicated and incubated with a mixture of H3, H2A, H4 which were labeled with [³H]acetic anhydride to 4,000 cpm/μg (25) for protease assay: the 60-μl reaction mixture contained 10 mM Tris, pH 7.5, 15 μg chromatin, 1 μg of ³H-histones (4,000 cpm), and 10% dimethyl sulfoxide. The mixture was incubated at 37°C for 24 h with and without inhibitors. After reaction, 60 μl of 50% TCA was added and radioactive acid-soluble peptides released were determined.

Under the conditions used above, the endogenous histones are not degraded,

but the chromatin-bound protease cleaves the exogenous acetylated histones at a slow rate.

For labeling chromatin with [³H]DFP in the presence of inhibitors, 10 μCi of [³H]DFP was mixed with 100 μg of chromatin in 0.2 ml of 10 mM Tris, pH 7.5. After incubation for 2 h at 37°C, the mixture was extensively dialyzed against 1% SDS before counting. Labeling was linear during the 2-h labeling period.

RESULTS AND DISCUSSION

There are many proteases which are inhibited by DFP as the result of phosphorylation of the active-site serine (5). The protease associated with chromatin prepared from various animal tissues is also inhibited by DFP (3, 4). Therefore, we first investigated the possible labeling of polytene chromosomes by incubation of salivary glands with [³H]DFP under physiological conditions. To insure that the DFP-labeled material is a protease, we used various protease inhibitors as competitors of DFP.

Autoradiography of chromosome spreads prepared by the standard 45% acetic acid squash technique (2) shows very little labeling of the chromosomes by [³H]DFP (Fig. 1). However, chromosomes prepared by squashing the salivary glands fixed with formaldehyde (see Materials and Methods) show extensive labeling by [³H]DFP (Fig. 2). These results suggest that the DFP-binding proteins are readily extracted by acetic acid during fixation. Acetic acid treatment also removes most of the histones and a significant amount of nonhistone proteins of the chromosome (6, 21) and the protease associated with mammalian tissue chromatin (3). Although the appearance of formaldehyde-fixed chromosomes (compare Fig. 1 with Figs. 2 and 3), the label appears to be more heavily associated with bands than with interbands (Fig. 2b). Labeling of nonchromosomal nuclear and cytoplasmic material is very light compared to labeling on the chromosomes (see arrows, Figure 2a). Labeling on the nucleolus is barely above background (see nu, Fig. 2a). The low labeling of cytoplasmic material could be caused by the extensive spreading of cytoplasm during squashing.

It is highly unlikely that the extensive labeling of chromosomes by [³H]DFP is caused by any relocation of cytoplasmic DFP-binding protein to the chromosome during the prepara-

FIGURE 1 Autoradiography of polytene chromosomes prepared by acetic acid procedure. Salivary glands were labeled for 30 min with [³H]DFP as described in Materials and Methods, and chromosome spreads were prepared by squashing the glands after fixing in 45% acetic acid. Exposure time, 3 wk. × 1,750.
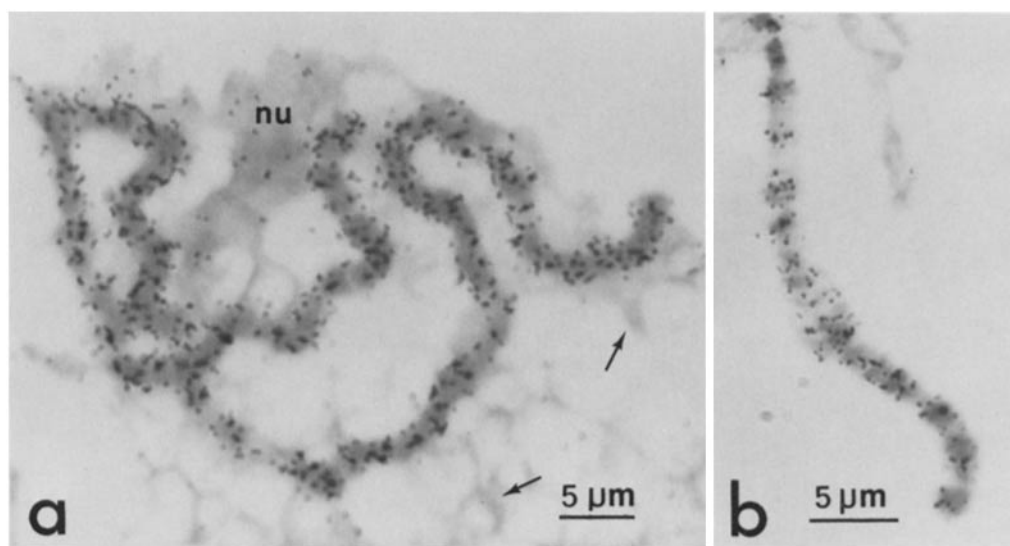

FIGURE 2 Autoradiography of polytene chromosomes prepared by formaldehyde-acetic acid procedure. Glands were labeled as indicated in legend to Fig. 1 and then fixed in formaldehyde. Chromosome spreads were prepared in formaldehyde-acetic acid as described in Materials and Methods. (a) Exposure time, 3 wk. nu, Nucleolus; arrows indicate nonchromosomal material. × 2,000. (b) Exposure time, 2 wk. × 2,500.

tion of the chromosome squash because the whole salivary glands were fixed with formaldehyde before squashing. Using this procedure it was shown that cyclic GMP, but not cyclic AMP, is associated with the heat-shock specific puffs (22). Both of these compounds are diffusible nucleotides, and it is highly unlikely that cyclic GMP, but not cyclic AMP, would associate with specific loci of chromosomes if they are relocated during the preparation of polytene chromosomes.

To see whether the labeling of polytene chromosomes by [³H]DFP was caused by the presence of a protease, salivary glands were preincubated with various protease inhibitors before exposing them to [³H]DFP. Control glands were preincubated in buffer without protease inhibitors. It was shown that

active site-directed irreversible inhibitors, although they do not necessarily interact with the serine, will prevent the phosphorylation of the active-site serine by DFP, perhaps by steric hinderance (19). Among the inhibitors tested, tosyl phenylalanine chloromethyl ketone (TPCK), an inhibitor for chymotrypsin-like protease (13, 16), prevents the labeling of the chromosomes almost completely at a concentration of 0.1 mM (compare Fig. 3 a and b) but tosyl lysine chloromethyl ketone (TLCK), an inhibitor for trypsin-like protease (16, 20), shows no inhibition of DFP labeling (data not shown). Phenylmethane sulfonylfluoride (PMSF) also reduces the labeling of the chromosome by [³H]DFP (Fig. 3 c), but the effect is less striking than with TPCK. Carbobenzoxyphenylalanine chlo-
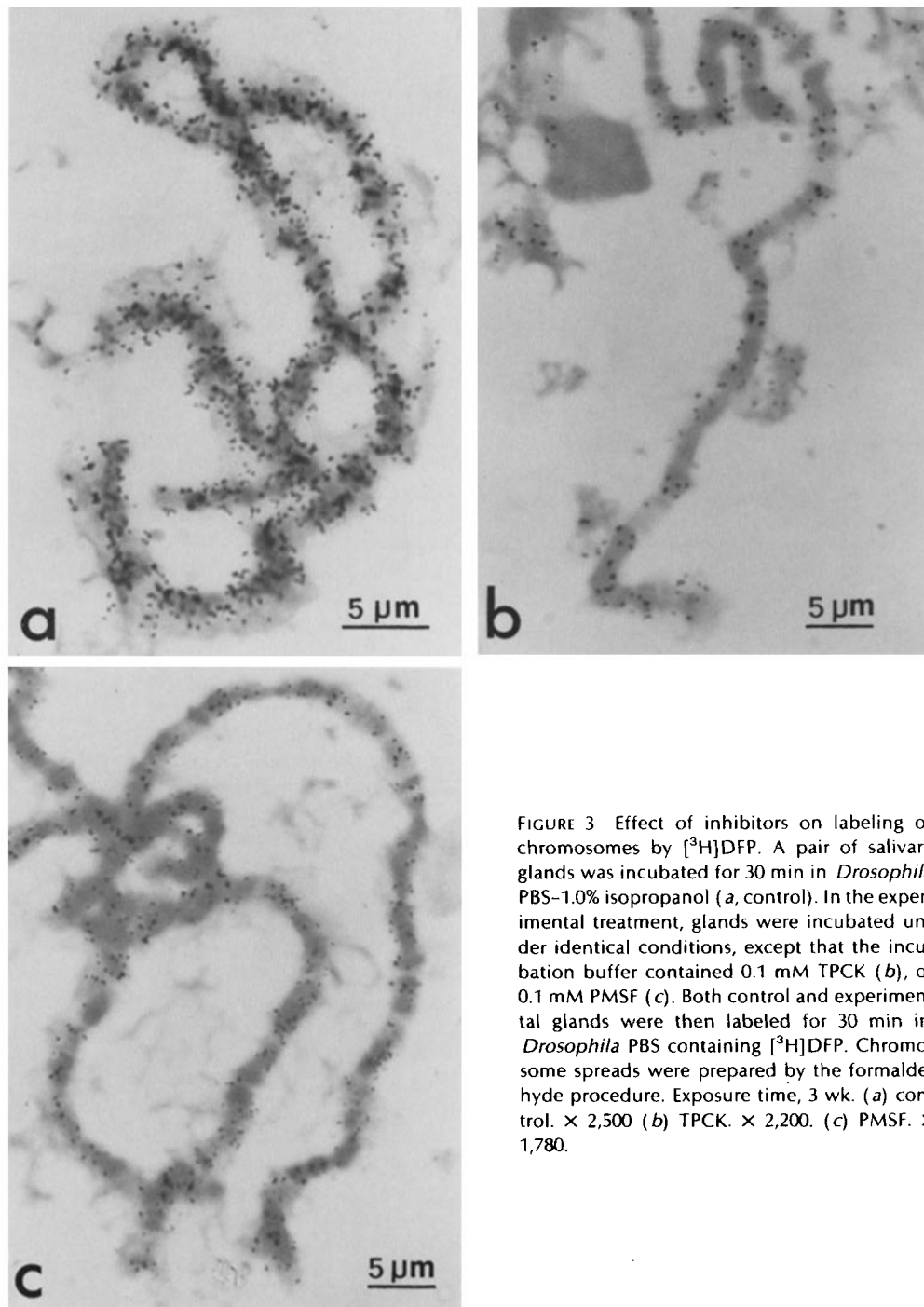

FIGURE 3   Effect of inhibitors on labeling of chromosomes by [³H]DFP. A pair of salivary glands was incubated for 30 min in *Drosophila* PBS-1.0% isopropanol (*a*, control). In the experimental treatment, glands were incubated under identical conditions, except that the incubation buffer contained 0.1 mM TPCK (*b*), or 0.1 mM PMSF (*c*). Both control and experimental glands were then labeled for 30 min in *Drosophila* PBS containing [³H]DFP. Chromosome spreads were prepared by the formaldehyde procedure. Exposure time, 3 wk. (*a*) control. × 2,500 (*b*) TPCK. × 2,200. (*c*) PMSF. × 1,780.

romethyl ketone (ZPCK) has no apparent effect (data not shown). Similar results were obtained with TPCK and other inhibitors when one lobe of a set of glands was incubated with inhibitor and the corresponding lobe without inhibitor. These results strongly suggest that [³H]DFP labels a chymotrypsin-like protease associated with polytene chromosomes.

The results shown here are not direct evidence for the association of a protease with chromosomes. However, the fact that the DFP labeling is inhibited by PMSF and TPCK and also that the substrate analogue, TPCK and TLCK, show selectivity in competition with DFP strongly suggests that DFP labels a chymotrypsin-like protease on the chromosomes. Also, the presence of a tightly bound protease in chromatin prepared from various mammalian tissues as well as from Drosophila embryo (see Table I) increases the likelihood of the presence of a protease with polytene chromosomes. As far as we can determine, there has been no report that TPCK or TLCK retards the DFP binding of nonprotease molecules such as cholinesterase (9) and nicotinamide deaminase (10), nor that DFP interferes with the reaction between nonprotease molecules, such as elongation factor Tu, and TPCK (17).

To obtain an estimate of the molecular weight of the DFP-binding proteins associated with polytene chromosomes, 20 salivary glands were pretreated with 0.1 mM TPCK or TLCK and subsequently labeled with [³H]DFP. The labeled glands were prepared for SDS gel electrophoresis of proteins. Whole salivary glands were used because of the low yield of nuclei from such a limited number of glands. The electrophoresis of total salivary gland proteins shows several DFP-binding proteins (Fig. 4). Only labeling of the 78,000-dalton protein was selectively inhibited by 0.1 mM TPCK but not by 0.1 mM TLCK, as in the case of the labeling of the chromosomes by [³H]DFP (see Fig. 3). The labeling of the 25,000-dalton protein was inhibited by both TPCK and TLCK. There are many serine proteases of 25,000 mol wt. Drosophila embryo chromatin contains proteolytic activity with trypsin-like specificity as shown in Table I; the labeling of this enzyme with [³H]DFP is inhibited by TLCK but not by TPCK. The mol wt of the protease associated with embryo chromatin appears to be 25,000 from [³H]DFP labeling experiments (not shown here). Although this enzyme appears to be different in type and molecular weight from that associated with the polytene chromosomes, the fact that Drosophila embryo chromatin and mammalian tissue chromatin (3) contain a protease reinforces the likelihood that the labeling of polytene chromosome by
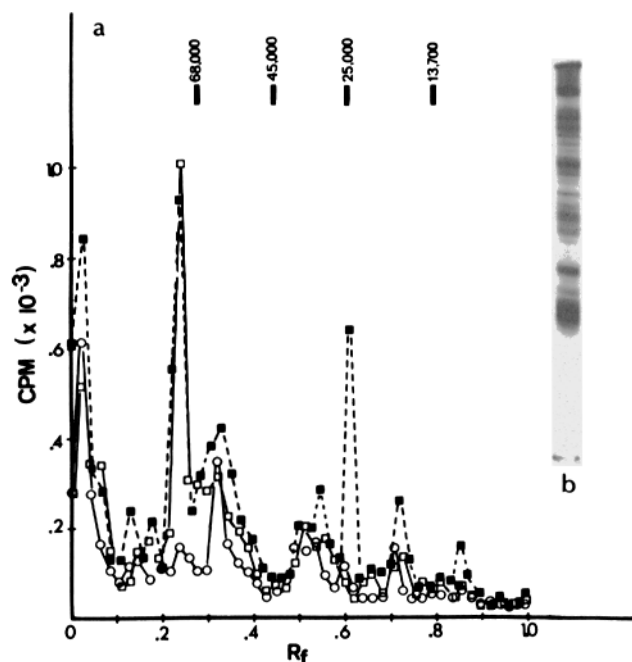
TABLE I

Inhibition of the Proteolytic Activity of Drosophila Embryo Chromatin

	% Inhibition	
Inhibitor	Degradation of [³H]histone	Labeling with [³H]DFP
None	0*	0‡
PMSF	80	85
TPCK	0	5
TLCK	92	90
ZPCK	30	Not tested

The concentration of inhibitors was 1 mM.
See Materials and Methods for experimental conditions.
Assays were conducted in duplicate.
* The radioactivity in the acid-soluble supernate of control chromatin was 520 cpm.
‡ 2,500 cpm.

FIGURE 4 SDS polyacrylamide gel electrophoresis of the total proteins of [³H]DFP-labeled salivary glands. Salivary glands were pretreated with 1.0% isopropanol (■), 0.1 mM TLCK in 1.0% isopropanol (□), and 0.1 mM TPCK in 1.0 isopropanol (O), and labeled with [³H]DFP as described in Materials and Methods. (a) Radioactivity of gel slices. (b) The gel stained with Coomassie Blue. (Inset).

[³H]DFP is caused by a bound serine protease, one with chymotrypsinlike specificity.

Next, we tested the possibility of inhibition of heat-shock-induced puffing by TPCK and PMSF as it was reasonable to hypothesize that gene activation may involve prior decondensation of supercoiled chromosomes through selective cleavage of H1 histone by the chromosome-associated protease. Incubation of chromatin (11, 12) or oligomeric nucleosomes (Y.-J Kim and C.-B. Chae, unpublished results) in the presence of 0.15 M NaCl, 1 mM MgCl₂, or 1 mM CaCl₂, which has been shown to cause supercoiling (23), causes degradation of H1 but not core histones by the chromatin- or nucleosome-associated protease. However, no degradation of histones including H1 occurs in low ionic strength buffer which relaxes chromatin structure (23). To test the possible effect of protease inhibitors on the heat-shock specific puffing, TPCK or PMSF was preincubated with salivary glands for 30 min to insure complete inhibition of the protease associated with polytene chromosomes before heat-shock treatment. However, TPCK and PMSF showed no apparent effect on the heat-shock specific puffing (data not shown). DFP was not tested because of its high toxicity and the possible hazard in handling millimolar concentrations. These results suggest that the function of the chromosome-associated protease may not be directly related to puff formation.

The extensive distribution of the protease on polytene chromosomes nevertheless suggests that this enzyme may have a role in the structure and/or function of these chromosomes through controlled cleavage and turnover of chromosomal proteins.

REFERENCES

1. Ashburner, M. 1970. Patterns of puffing activity in salivary gland chromosomes of Drosophila. V. Responses to environmental treatment. *Chromosoma (Berl.)*. 31:356–376.
2. Atherton, D. D., and J. G. Gall. 1972. Salivary gland squashes for *in situ* hybridization studies. *Dros. Int. Serv.* 49:131–133.
3. Carter, D. B., P. H. Efird, and C.-B. Chae. 1976. Chromatin-bound protease: ³H diisopropylfluorophosphate labeling of chromatin. *Biochemistry.* 15:2603–2607.
4. Carter, D. B., P. H. Efird, and C.-B. Chae. 1978. Chromatin-bound protease and their inhibitors. *Methods Cell Biol.* 19:175–190.
5. Cohen, J. A., R. A. Oosterbaan, and S. F. Berends. 1967. Organophorus compounds. *Methods Enzymol.* 11:686–705.
6. Dick, C., and W. Johns. 1968. The effect of two acetic acid containing fixatives on the histone content of calf thymus deoxyribonucleoprotein and calf thymus tissue. *Exp. Cell Res.* 51:626–632.
7. Elgin, S. C. R., and L. E. Hood. 1973. Chromosomal proteins of Drosophila embryos. *Biochemistry.* 12:4984–4991.
8. Ephrussi, B., and G. W. Beadle. 1936. A technique of transplantation for Drosophila. *Am. Nat.* 70:218–225.
9. Froede, H. C., and I. B. Wilson. 1971. Acetylcholinesterase. *In* The Enzymes. P. D. Boyer, editor. Academic Press, Inc., New York. 5:87–114.
10. Gillam, S. S., J. G. Watson, and S. Chaykin. 1973. Nicotinamide deamidase from rabbit liver. III. Inhibition and sedimentation studies. *Arch. Biochem. Biophys.* 157:268–284.
11. Hale, P. M. 1978. Cleavage of H1 Histone by an Endogenous Chromosomal Protease Under Unique Conditions. *Fed. Proc.* 37:1640.
12. Hale, P. M. 1978. Endogenous Chromosomal Proteolysis of Histones, Ph.D. Thesis, University of North Carolina, Chapel Hill, N. C.
13. Kezdy, F. J., A. Thomson, and M. L. Bender. 1967. Studies on the reaction of chymotrypsin and L-1-chloro-3-tosylamido-4-phenyl-2-butanone. *J. Am. Chem. Soc.* 89:1004–1009.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
15. Pierce, D. A., and J. C. Lucchesi. 1980. Dosage compensation of X-linked heat-shock puffs in *Drosophila pseudoobscura. Chromosoma (Berl.).* 76:245–254.
16. Powers, J. C. 1977. Haloketone inhibitors of proteolytic enzymes. *In* Chemistry and Biochemistry of Amino Acids, Peptides and Proteins. B. Weinstein, editor. Marcel Dekker, New York. 4:65–178.
17. Richman, N., and J. W. Bodley. 1973. Irreversible inhibition of the interaction between elongation factor tu and phenylalanine transfer ribonucleic acid by L-1-tosylamido-2-phenylethyl chloromethyl ketone. *J. Biol. Chem.* 248:381–383.
18. Ritossa, F. 1974. Experimental activation of specific loci in polytene chromosomes of Drosophila. *Exp. Cell Res.* 35:601–607.
19. Shaw, E. 1970. Chemical modification by active-site-directed reagents. *In* The Enzymes. P. D. Boyer, editor. Academic Press, Inc., New York. 1:91–146.
20. Shaw, E., M. Mares-Guia, and W. Cohen. 1965. Evidence for an active-center histidine in trypsin through use of a specific reagent, 1-chloro-3-tosylamido-7-amino-2-heptanone, the chloromethyl ketone derived from N$^\alpha$-tosyl-L-lysine. *Biochemistry.* 4:2219–2224.
21. Silver, L. M., and S. C. R. Elgin. 1978. Immunological analysis of protein distributions in Drosophila polytene *chromosomes. In* The Cell Nucleus. Harris Busch, editor. Academic Press, Inc., New York. 4 (Pt. B):215–262.
22. Spruill, W. A., D. R. Hurwitz, J. C. Lucchesi, and A. L. Steiner. 1978. Association of cyclic GMP with gene expression of polytene chromosomes of *Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A.* 75:1480–1484.
23. Thoma, F., T. H. Koller, and A. Klug. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J. Cell Biol.* 83:403–427.
24. Tissieres, A., H. K. Mitchell, and U. M. Tracy. 1974. Protein synthesis in salivary glands of *Drosophila melanogaster:* relation to chromosome puffs. *J. Mol. Biol.* 84:389–398.
25. Wyrick, S., Y-J. Kim, K. Ishaq, and C.-B. Chae. 1979. Synthesis of active site-directed organometallic irreversible protease inhibitors. *Biochim. Biophys. Acta.* 568:11–18.