

A Cytological Approach to the Ordering of Events in Gene Activation Using the *Sgs-4* Locus of *Drosophila melanogaster*

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ABSTRACT The polytene chromosomes of *Drosophila* strains that differ in the synthesis of the major salivary gland glue protein *sgs-4* were examined by indirect immunofluorescence using antisera to several nonhistone chromosomal proteins. The Oregon-R X chromosome, which produces *sgs-4* messenger RNA, showed a strong fluorescent band at locus 3C11-12 when stained with anti-RNA polymerase II, whereas the null mutant Berkeley 1 failed to exhibit fluorescence at that locus. The presence of another antigen (Band 2), normally associated with developmentally active loci, was clearly evident at locus 3C11-12 of both transcriptionally competent and null strains, indicating that the association of Band 2 antigen with the chromatin is an event independent of RNA polymerase II binding. Antibodies directed against *Drosophila* topoisomerase I stained 3C11-12 in the *Sgs-4*⁺ (wild-type) strain brightly, but gave significantly less staining in the null strain. This indicates that the high concentrations of topoisomerase I seen at active loci are closely associated with the transcriptional event. In some of these analyses, we have made use of flies heterozygous for the wild-type and null alleles in order to make internally controlled comparisons. The results suggest that this type of analysis will enable conclusions to be drawn concerning the interdependence and order of action of chromosomal proteins involved in developmental gene activation.

The indirect immunofluorescence technique (1, 23) allows a visual assay for the presence or absence of protein molecules on polytene chromosomes, and can therefore be used to examine macromolecular differences between active and inactive chromatin from cytological preparations. The study of the association of nonhistone chromosomal (NHC)¹ proteins with active chromatin and their temporal binding order should eventually lead to a hierarchical picture of events in gene activation. Even better than the use of progressively staged larvae in exploring the course of such biochemical changes is the use of well-characterized variants. Such variants might show altered programs of macromolecular association, detectable by the immunofluorescent assay, leading to alteration of gene expression.

The *Sgs-4* locus at 3C11-12 in *Drosophila melanogaster* encodes a peptide component of the larval glue which is secreted by the third instar larvae at the beginning of pupariation (3, 8). Chromatin structure analyses of the wild-type and several well-characterized variant alleles have revealed a set of tissue-specific deoxyribonuclease (DNase) I hypersensi-

tive sites associated with the wild-type locus, some or all of which are abrogated in the variant lines (22). A wide variety of different genes subject to different programs of regulation have been shown to possess specific DNase I hypersensitive sites, generally at or near their 5' ends (see reference 5 for review). It has been suggested that the formation of such sites might be necessary but not sufficient for transcription. One goal of this study is to clarify the temporal position of DNase I hypersensitive sites among several biochemical interactions associated with developmental gene activity.

A comparison of several different NHC proteins which are found to be present at developmentally active and/or inducible loci has been made at the *Sgs-4* locus in a wild-type (*Sgs-4*⁺) and a variant, Berkeley 1 (BER-1), strain. The *Sgs-4* locus is inactive in the BER-1 flies. As *Sgs-4* appears to be the only transcript encoded at this locus (in 16–20 kilobase-pair [kb] of DNA) (17, 18), it is reasonable to correlate the structure of the chromomere with transcription of this particular gene. Antibody probes used include an anti-*Drosophila* RNA polymerase II (6, 20), anti-*Drosophila* topoisomerase I,² and

¹ Abbreviations used in this paper: BER-1, Berkeley 1 variant line; DNase, deoxyribonuclease; NHC, nonhistone chromosomal; SSC, saline-sodium citrate; w^{m4}, In(1)w^{m4} chromosome.

² Fleischmann, G., G. Pflugfelder, E. K. Steiner, K. Javaherian, G. C. Howard, J. C. Wang, and S. C. R. Elgin, manuscript submitted for publication.

anti-Band 2 serum (14), which was made against a protein fraction preferentially released by limited DNase I digestion of *Drosophila* embryo nuclei. We show the specific association of these proteins at wild type and variant *Sgs-4*, and discuss the general utility of this technique as an approach to defining the hierarchy of changes occurring in chromatin structure that lead to developmental gene activation.

MATERIALS AND METHODS

Drosophila Stocks: Oregon R (obtained from D. Hogness) and In(1)^{w^{m4}} (obtained from I. Duncan) are described in reference 11. The In(1)^{w^{m4}} chromosome is marked with *y w m f*. BER-1 (obtained from S. Beckendorf) is a variant line which synthesizes no detectable *sgs-4* messenger RNA (19). In(1)^{w^{m4}} × BER-1 hybrid females were generated by crossing males bearing the In(1)^{w^{m4}} chromosome to virgin BER-1 females. *ry⁵⁰⁶* (obtained from D. Hartl) carries an *Sgs-4⁺* allele and was used as a control in the RNA blot experiment. Flies were cultured in half-pint plastic bottles at 18°C, using a cornmeal-based medium supplemented with dried bakers yeast.

Salivary Gland Squashes: Early third instar larvae were selected, because the *Sgs-4* gene is active at this developmental stage. To insure that the larvae were at the appropriate developmental stage (puffing stage 1 or 2 according to reference 2), we examined the chromosomes after squashing for the presence of the developmental puff at 68C. The absence of puffs at 74EF or 75B was also used to determine that a larva had not yet reached stage 3. Glands were squashed (according to reference 24), by using either the acetic acid fixation or the formaldehyde fixation procedures. Chromosomes to be stained with anti-*Drosophila* RNA polymerase II or anti-*Drosophila* topoisomerase I sera were from glands squashed in 45% acetic acid–3.3% formaldehyde. Those intended for assay of Band 2 antigen were from glands fixed with 2% formaldehyde before squashing as above. Such fixation is necessary because the major antigen of Band 2 is acid extractable. Control studies with a variety of fixation techniques as well as positive results in previous studies support the conclusion that the fixation procedures used here are suitable for the antigens under study (6, 14, 20, 24; footnote 2).

Immunofluorescent Staining of Polytene Chromosomes: Indirect immunofluorescent staining was performed according to reference 25. Rabbit anti-*Drosophila* RNA polymerase II serum (26) was stored frozen at –20°C in 1:1 ethylene glycol and used at dilutions of 1:20 and 1:40. Rabbit anti-*Drosophila* topoisomerase I antibody was affinity-purified from serum,² stored in a 30% glycerol solution, and used at a dilution of 3:2 in a 10 mg/ml solution of bovine γ -globulin (Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline. Anti-Band 2 serum (13) was used at dilutions of 1:15 and 1:20. Fluorescent visualization was by secondary staining with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN) used at a dilution of 1:20.

RNA Analysis: Total nucleic acids were prepared essentially according to reference 17. Eight late third-instar larvae were homogenized for 1 min in a motor-driven glass-TEFLON homogenizer in 200 μ l of phenol plus 200 μ l of RNA extraction buffer (100 mM Tris–HCl, pH 8.0; 100 mM NaCl; 20 mM ethylenediaminetetraacetate; 1% sodium lauryl sarkosine; 0.04 mg/ml polyvinyl sulfate). The homogenate was centrifuged in a Brinkmann Instruments, Inc. microfuge (Westbury, NJ) for 5 min, and the aqueous phase was extracted twice more with phenol and once with chloroform, all at room temperature. The aqueous phase was then made 0.2 M with respect to NaCl and the nucleic acids were precipitated with two volumes of absolute ethanol and recovered by spinning 10 min in the microfuge.

The dried precipitate was taken up in 60 μ l of RNA sample buffer (gel-running buffer made up to 50% formamide, 2.2 M formaldehyde; gel-running buffer is 20 mM morpholinopropane sulfonate, pH 7.0; 50 mM sodium acetate, pH 7.0; 1 mM ethylenediaminetetraacetate) and heated for 15 min at 60°C. 10 μ l of sample were mixed with 4 μ l of loading solution (0.75% bromophenol blue; 50% sucrose in gel-running buffer) and loaded onto a 1.5% agarose formaldehyde gel (1.5% agarose [Seakem]; 6% formaldehyde; 1 μ g/ml ethidium bromide in gel-running buffer; reference 10). The gel was electrophoresed at room temperature in a submarine apparatus at 40 V until the marker dye reached the bottom of the gel. The gel was then soaked in 10 × SSC (saline–

sodium citrate [SSC] is 0.15 M NaCl; 0.015 M sodium citrate, pH 7.0) for 30 min with one change and blotted to nitrocellulose paper in 10 × SSC (25).

The nitrocellulose filter was then baked for 3 h at 70°C in vacuo, soaked for 3 h in hybridization solution (4 × SSC; 40% formamide; 100 mM Tris–HCl, pH 7.4; 0.5% sodium dodecyl sulfate; 1 × Denhardt's solution) (4) at 42°C, then hybridized overnight in 50 μ g/ml sheared-salmon sperm DNA and 200 ng of ³²P-labeled Dm2151 DNA. Dm2151 is a phage lambda clone which includes the transcribed *sgs-4* sequences (17). DNA was labeled to high specific activity by nick translation essentially according to reference 13. The hybridized blot was then washed in 2 × SSC–0.25% SDS for 1 h at room temperature, dried, and exposed overnight to XAR-5 (Eastman Kodak Co., Rochester, NY) x-ray film at –80°C using a Cronex lightning-plus (Corning Glass Works, Corning, NY) intensifying screen.

RESULTS

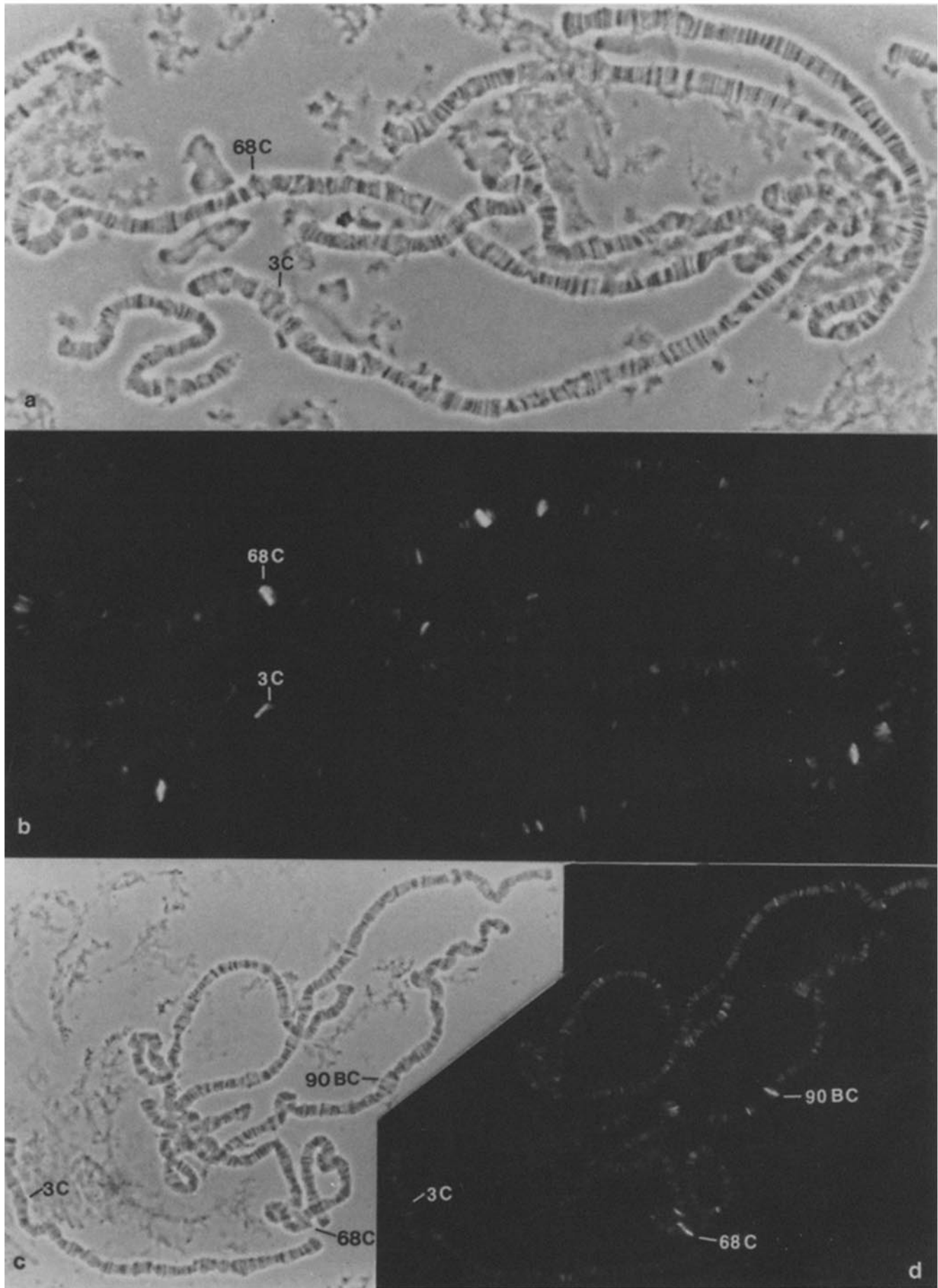
When assayed for the presence of *Drosophila* RNA polymerase II, the chromosomes of the *Sgs-4⁺* Oregon-R strain showed an intensely fluorescent band at locus 3C11-12 during the early stages of the third instar larval period when this gene is known to be active (Fig. 1). The virtual absence of fluorescence at 3C11-12 in chromosomes from the null variant BER-1 is apparent by comparison.

In making a comparison of this type for a developmentally regulated gene, it is of critical importance to use precisely staged larvae. To insure that both chromosomes examined are at precisely the same stage, we stained squashes of salivary glands from BER-1 × In(1)^{w^{m4}} (abbreviated here “w^{m4}”) hybrid female larvae. The choice of the w^{m4} chromosome permits cytological identification of the wild-type *Sgs-4⁺*-bearing chromosome arm, because the continuity of the X euchromatin is interrupted in the inversion by heterochromatin which can be broken in squashing. In addition, the breakage of the distal w^{m4} heterochromatin is frequently accompanied by asynapsis of the X chromatids for some distance proximal to the site of breakage, thus physically separating the wild-type and variant alleles and permitting their unambiguous identification. Fig. 2 shows an example of such a squash stained with anti-*Drosophila* RNA polymerase II antiserum. Again, bright fluorescence is observed only at the transcriptionally active *Sgs-4* site. That the w^{m4} chromosome indeed synthesizes *sgs-4* mRNA is demonstrated by the identification of *sgs-4* mRNA in w^{m4} males (Fig. 3).

In contrast to proteins of the transcriptional machinery, which might reasonably be expected to associate only with transcriptionally active loci, Band 2 protein has previously been found to associate with most loci active in salivary glands at some time during larval development (14). This pattern indicates prior association of the antigen with activatable loci. As *Sgs-4* is normally active in third instar salivary glands, it was of interest to determine whether Band 2 protein associates with this locus in the BER-1 variant. Fig. 4 shows a comparison of anti-Band 2 staining at *Sgs-4* in Oregon-R and BER-1; bright fluorescence is observed at 3C11-12 in squashes from both lines.

Recent work has shown a similarity in the distributions of topoisomerase I and RNA polymerase II in *Drosophila* polytene chromosomes.² We have compared the relative amounts of topoisomerase I at the wild-type and null *Sgs-4* locus using this immunofluorescence assay. Fig. 5 shows polytene chro-

FIGURE 1 Distribution pattern of DNA-dependent RNA polymerase II in early third instar salivary glands of Oregon-R (a and b) and BER-1 (c and d). a and c, phase-contrast; b and d, fluorescent micrographs.



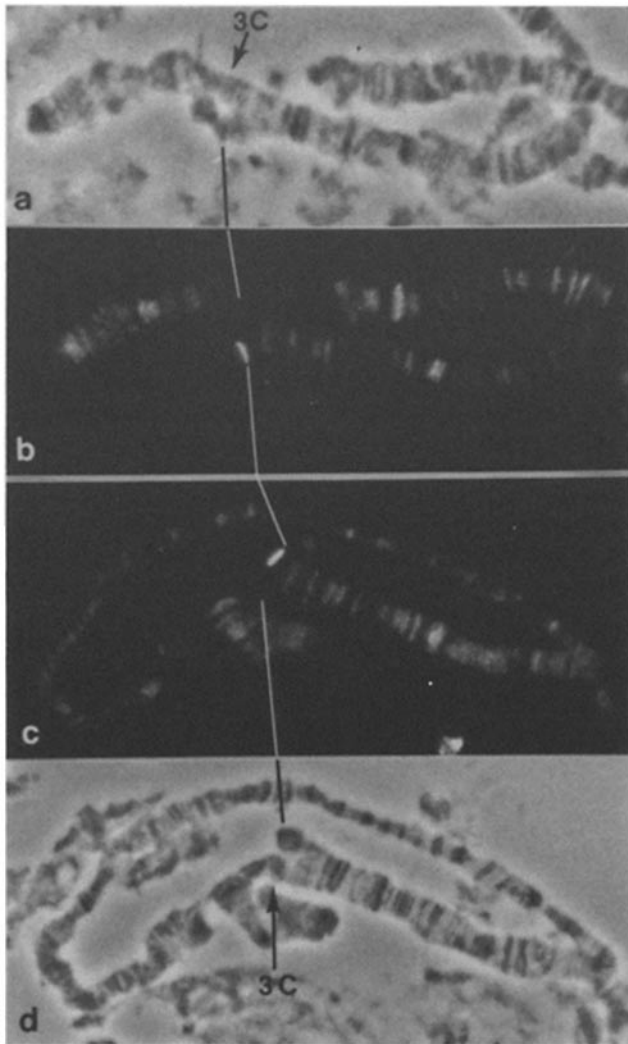


FIGURE 2 Comparison of the distribution of RNA polymerase II at the 3C region of the X chromosome from hybrid $In(1)w^{m4} \times BER-1$ female larvae. (a and b) The straight and mostly synapsed tips of the X arms. (c and d) A typically looped X chromosome, resulting from synapsis of the inverted and noninverted X chromosome arms.

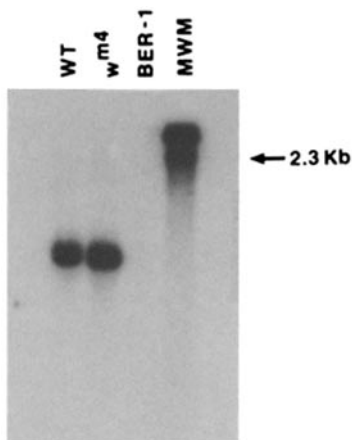


FIGURE 3 Assay for *Sgs-4* mRNA from the $In(1)w^{m4}$ chromosome. Total nucleic acids were prepared from ry^{506} (WT), $In(1)w^{m4}$ males (w^{m4}), and $BER-1$ late third-instar larvae ($BER-1$), electrophoresed in a 1.5% formaldehyde gel, and blotted to nitrocellulose (see Materials and Methods). The blot was probed with Dm2151 (18). Only males from the $In(1)w^{m4}$ stock were used. Size marker (MWM) is phage lambda DNA cut with Hind III.

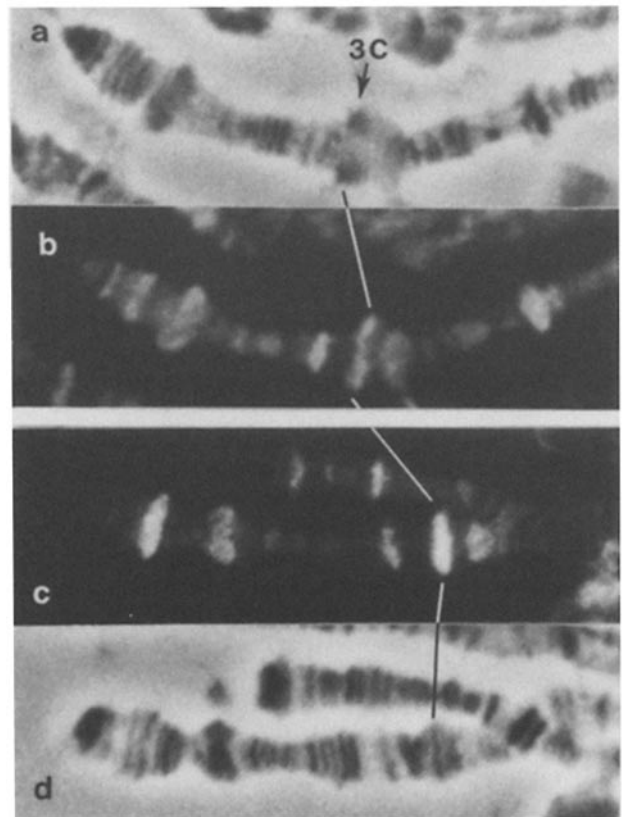


FIGURE 4 Phase-contrast (a and d) and fluorescent (b and c) micrographs of locus 3C in formaldehyde-fixed Oregon-R (a and b) and $BER-1$ (c and d) chromosomes stained using anti-Band 2 serum.

mosome squashes from a $BER-1 \times w^{m4}$ heterozygous female, stained with anti-topoisomerase 1 serum. A strong fluorescence, obvious at 3C11-12 in the w^{m4} chromosome is clearly absent at the same site in the $BER-1$ chromosome.

DISCUSSION

Sgs-4, a gene whose activity is restricted to the third instar larval salivary gland, has been mapped cytologically (8) and genetically (9, 15), and several variants have been characterized (3, 16, 19, 22). A set of five DNase I hypersensitive sites are associated with the wild-type *Sgs-4* gene in a tissue- and developmentally specific fashion. These sites are absent in the *Sgs-4* null variant $BER-1$, despite the fact that the DNA sequence associated with three of the sites, and the TATA box sequence, remain essentially unaltered (22). Using an indirect immunofluorescence assay, we have observed the association of three NHC proteins at 3C11-12 in polytene chromosomes from *Sgs-4* wild-type lines and the $BER-1$ variant, in an attempt to assign a temporal order to biochemical events associated with developmental gene activity and relate them to this change in the chromatin structure.

In previous experiments, RNA polymerase II has been shown to be associated with polytene chromosome puffs in general (6, 20) and with the wild-type 3C11-12 puff in particular (6, 7). By contrast, our data show the null variant $BER-1$ shows only background levels of fluorescence at this locus. Given the absence of other active genes within the 16–20 kb surrounding *Sgs-4*, this assay should be specific for *Sgs-4* (15,

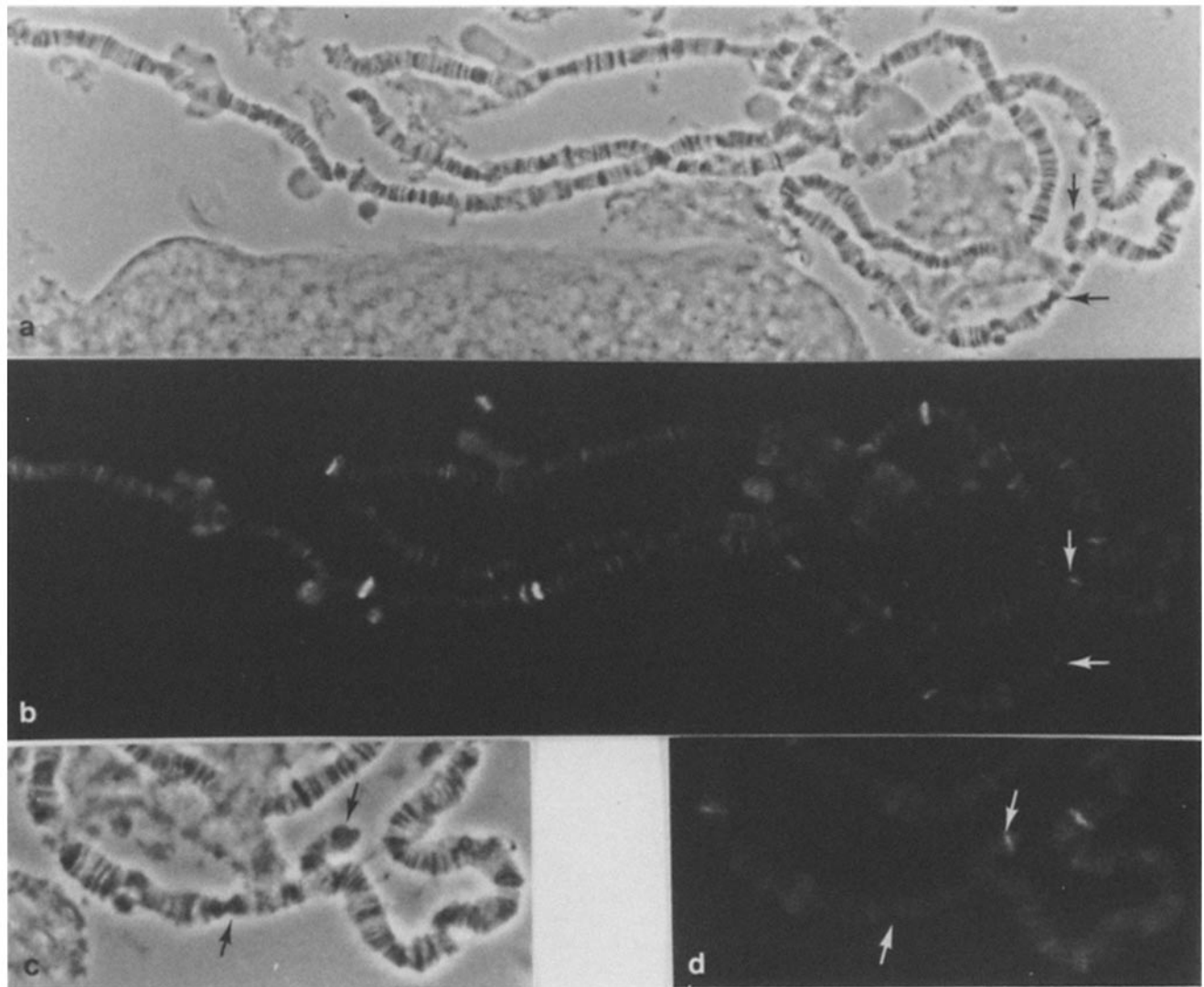


FIGURE 5 Distribution pattern of topoisomerase 1 in an early third-instar $w^{m4} \times \text{BER-1}$ female larvae. (c and d) A detail of the partially asynapsed X chromosome from a and b. Arrows indicate position of locus 3C11-12.

18). We cannot exclude the possibility of low levels of polymerase II loading at 3C11-12 in BER-1; in fact, only background levels of fluorescence are detectable at 3C11-12 in the Hikone-R variant (7), which is transcriptionally active, albeit at $<2\%$ of wild type levels (19). A dramatic reduction in fluorescence at both of these variant alleles is consistent with the conclusion that the level of enzyme is correlated with the transcription level.

Like RNA polymerase II, topoisomerase I is a NHC protein whose biochemical activity is well characterized; however, the function of topoisomerase I in eucaryotic chromatin is as yet unknown. Because of its ability to relax supercoiled DNA, one might infer a role for topoisomerase I in uncoiling condensed, inactive chromatin for transcription and/or in facilitating the transcription process. Indeed, a recent study shows similarities in the distribution of topoisomerase I and RNA polymerase II in polytene chromosomes.² When the presence of topoisomerase I is assayed by indirect immunofluorescence at 3C11-12 in $w^{m4} \times \text{BER-1}$ hybrid female larval salivary gland chromosomes, bright fluorescence is found only at the

wild-type chromosome arm.³ Thus, like RNA polymerase II, topoisomerase I is apparently closely associated with transcription. While it is not possible to obtain quantitative information from this technique, it can be observed that the fluorescence intensities at 3C11-12 in $Sgs-4^+$ chromosomes are similar when stained for topoisomerase I or RNA polymerase II (compare Figs. 1 and 5). This would suggest the presence of topoisomerase I at the active locus at a high level,

³ The use of the w^{m4} chromosome in these analyses provides an internal control for proper staging of the $Sgs-4$ locus, as well as facilitating direct comparison of relative fluorescence intensities between the wild-type and variant alleles. Although the w^{m4} rearrangement does variegate for the white locus, there is no evidence for variegation extending to the more distal (with respect to the breakpoint) $Sgs-4$ locus. Furthermore, northern analysis (Fig. 3) shows no obvious reduction in levels of $sgs-4$ message suggestive of significant variegation. Finally, the w^{m4} $Sgs-4$ allele behaved consistently in all of our experiments (four separate experiments, 23 chromosome sets examined), suggesting no detectable levels of variegation at this locus under our conditions.

similar to that of RNA polymerase II. It is likely that small amounts of protein (i.e., one to two molecules per haploid genome) could not be detected by this test. Consequently, one would not exclude the possibility that topoisomerase I at such low levels is present at *Sgs-4* in BER-1 flies, required for earlier steps in the activation process.

Band 2 protein was initially identified as one of a class of NHC proteins released from *Drosophila* embryo chromatin by DNase I digestion, and it is found to be associated with most loci known to be active at some time in third instar larval salivary glands independent of their transcriptional state. The present experiments have shown Band 2 protein to be associated with the BER-1 *Sgs-4* locus. It is possible that Band 2 protein acts *in vivo* as a potentiator of gene activation, in contrast to proteins directly involved in the transcriptional machinery. The lesions that inactivate *Sgs-4* in the BER-1 variant and abrogate the pattern of DNase I hypersensitive sites associated with the wild-type gene have clearly left intact the signals involved in Band 2 protein binding.

While an absence of DNase I hypersensitive sites at the *Sgs-4* locus in BER-1 is correlated with an absence of transcription, DNase I hypersensitive sites have been found 5' to transcriptionally inactive genes (12, 27, 28). It has been proposed (5) that the formation of 5' DNase I hypersensitive sites is necessary but not sufficient for transcription *in vivo*. The preceding work would suggest that the association of Band 2 protein may be another in this class of chromatin events. Further work, including the possible use of mutations created *in vitro* and reintroduced into the genome by germ line transformation (21), will be necessary to fully characterize the requirements for Band 2 protein binding, and its role in developmental gene activation. This approach should allow us to identify requirements for the association of NHC proteins with a given locus, eventually establishing a hierarchy of events required for gene expression.

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