Proximity-Dependent Enhancement of *Sgs-4* Gene Expression in D. melanogaster

J. Stephen Kornher and Douglas Brutlag
Department of Biochemistry
Stanford University Medical Center
Stanford, California 94305

Summary

A weakly expressed allele of *Sgs-4* obtained from the Kochi strain of D. melanogaster (*Sgs-4^K*) increases 4-fold in levels of accumulated transcript when paired with a wild-type (Oregon-R) *Sgs-4* allele (*Sgs-4^{ORE}*) and increases 9-fold when paired with a duplication of the wild-type *Sgs-4* allele *Confluens* (*Sgs-4^{Co}*). There is no enhancement of expression when *Sgs-4^K* is paired with the *Sgs-4* null allele Ber-I (*Sgs-4^{BER}*) or with another weakly expressed *Sgs-4* allele, Hikone-R (*Sgs-4^{HIK}*); there is minimal enhancement when chromosome pairing is disrupted near the *Sgs-4* locus by rearrangement of the wild-type *Sgs-4* allele. Cytological analysis of third instar salivary gland nuclei from *Kochi/Confluens* heterozygotes suggests that complete pairing is not essential for *Sgs-4^K* enhancement. Interactions between homologous loci that could produce a *trans* enhancement effect are discussed and a hypothesis is formulated to test the significance of specific upstream sequences from both alleles in expression enhancement.

Introduction

In the somatic cells of most insects, homologous chromosomes tend to associate with each other or to pair (Metz, 1916). This phenomenon has been observed at various stages of the cell cycle, with the best-known example being the precise pairing of chromosome homologs in the polytene nuclei of the fruit fly Drosophila melanogaster. Although the significance of homolog pairing is not understood, cytological evidence in Drosophila suggests that disruptions in homolog pairing can influence the expression of genes located within the pairing-disrupted chromosome segment.

Cytological studies of puff patterns in the polytene chromosomes of interstrain heterozygotes (Ashburner, 1970) indicate that an unpuffed locus can be induced to puff when paired with a homologous locus that is puffed. This puff induction can be eliminated by disrupting the homolog pairing at the puff site through chromosomal rearrangement. Korge's studies of *Sgs-4* (1977, 1981), a sex-linked gene expressed in the larval salivary gland, demonstrate that certain weakly expressed *Sgs-4* alleles can be induced to puff when paired with *Sgs-4^{ORE}*, a strongly expressed *Sgs-4* allele. An *Sgs-4* protein that was not detected in larvae homozygous for the weakly expressed allele accumulated in the salivary gland lumen of these heterozygotes. This enhancement effect was eliminated when, as a result of rearrangement, chromosome homologs were unpaired at the *Sgs-4* locus.

The *Sgs-4* locus is a useful marker for measuring pairing-dependent enhancement effects. It is expressed in polytene cells, making cytological analysis possible. Its expression is pronounced and occurs when the nuclei are maximally polytene, so cytology and biochemistry can be applied to the same developmental stage. The *Sgs-4* gene has been isolated and characterized from several strains (Muskavitch and Hogness, 1980, 1982). *Sgs-4* RNA electrophoretic variants are available, with transcripts ranging in size from 0.9 kb to 2.7 kb. Finally, there is a broad range of interstrain *Sgs-4* expression: from a viable null strain (Ber-I) to a tandem duplication containing the *Sgs-4* locus (*Confluens*), which accumulates *Sgs-4* transcript to twice the levels attained by Oregon-R.

We describe a study of enhancement effects on the transcription of the *Sgs-4* gene. Heterozygotes for various *Sgs-4* alleles were constructed. In the majority of these one copy of a weakly expressed *Sgs-4* allele and one copy of a strongly expressed *Sgs-4* allele were juxtaposed. In all the heterozygotes analyzed the *Sgs-4* transcripts differed sufficiently in size to be resolved electrophoretically and characterized by Northern analysis utilizing a cloned *Sgs-4* probe. By screening for combinations of *Sgs-4* alleles that resulted in enhancement of the weaker allele's expression we found that making weak *Sgs-4* alleles from the Kochi and Hikone-R strains heterozygous with *Confluens* enhanced their expression 9-fold and 2-fold, respectively. In addition, we found a proportionality between the amount of *Sgs-4^K* expression and the number of strongly expressed *Sgs-4* alleles juxtaposed with it. Finally, *Sgs-4^K* expression was minimal when chromosomal rearrangements bearing strongly expressed *Sgs-4* alleles with a break point in the vicinity of *Sgs-4* were made heterozygous with Kochi. We consider these results in the context of a working hypothesis that addresses the molecular basis for such enhancement effects.

Results

Kochi *Sgs-4* Expression Is Enhanced When Paired with Strongly Expressed *Sgs-4* Alleles

Utilizing a cloned *Sgs-4* probe (Dm1513, courtesy of M. Muskavitch), we screened several weakly expressed *Sgs-4* alleles for increases in levels of accumulated transcript when made heterozygous with *Sgs-4^{ORE}*. To provide a measure of normal expression for a particular *Sgs-4* allele, females homozygous for that allele were included in the analysis. The strains assayed for expression enhancement were Ber-I, Kochi, Hikone-R, Seto, S8D, and Samarkand. Third instar salivary gland RNA from each of these strains was assayed in a pairwise fashion with RNA obtained from strains heterozygous with Oregon-R. Of the *Sgs-4* alleles assayed in the Oregon-R screen, only *Sgs-4^K* showed enhanced expression when made heterozy-
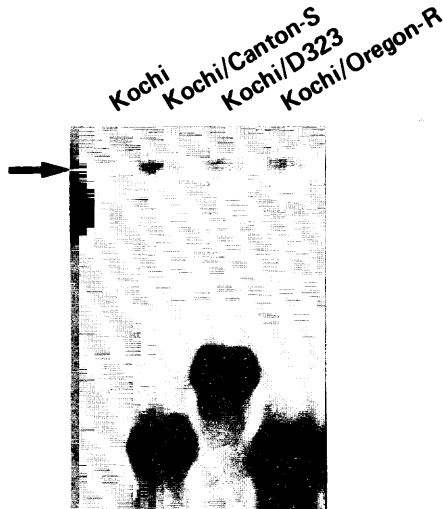
Figure 1. Similar Enhancement of Kochi Sgs-4 Expression Occurs in the Presence of D323, Canton-S, or Oregon-R Alleles

Total RNA was isolated from third instar larval salivary glands. A measured amount of this RNA, based on absorbance at 260 nm, was glyoxylated, electrophoresed through a neutral agarose gel, blotted onto nitrocellulose, and probed with the Sgs-4 clone Dm1513. The arrow indicates the Kochi Sgs-4 RNA common to all lanes. The remaining RNAs are derived from the strongly expressed Sgs-4 alleles used in the screen.

gous with Sgs-4^{ORE}. When the data are corrected for the Sgs-4^K dose in the homozygote and heterozygote, expression in the heterozygote is 4-fold that in the Kochi female control. When Sgs-4 alleles that attain 40% to 50% Oregon-R Sgs-4 transcript levels (Canton-S, D323) were juxtaposed with the Kochi allele a similar enhancement of Sgs-4^K expression was observed (see Figure 1 and Table 1).

Enhancement of Kochi Sgs-4 Expression Is Twice As Great When Confluens Is Substituted for Oregon-R

In a second screen, Confluens was substituted for Oregon-R, most of the weak Sgs-4 alleles were retested, and enhancement was quantified as described in the legend to Table 1. Confluens is a tandem duplication of the 3C4-5; 3D6-E1 chromosome region (Lindsley and Grell, 1968) that contains the Sgs-4 gene (3C11-12). This strain accumulates twice the levels of Sgs-4 transcript found in Oregon-R (data not shown).

This second screen uncovered two strains with enhanced Sgs-4 expression: Kochi, with 9-fold enhancement, and Hikone-R, with 2-fold enhancement. The results of the Oregon-R and Confluens screens are summarized in Table 1. These data suggest that the enhancement of Sgs-4^K expression is proportional to the dose of strongly expressed Sgs-4 allele present in the heterozygote (see Figure 2).

Null or Weak Sgs-4 Alleles Do Not Influence Kochi Sgs-4 Expression

The Kochi, Seto, and Hikone-R strains comprise a group of weakly expressed Sgs-4 alleles (~2% of Oregon-R). They are highly conserved over an ~600 bp stretch of upstream DNA sequence. Among several differences at the 5' end, the three strains have an upstream deletion at position −305 to −356 in common (Muskavitch and Hogness, 1982). Heterozygotes for Kochi and either Hikone-R or Seto do not enhance the expression of either hypomorphic allele. Sgs-4^K expression is also unaffected when heterozygous with Ber-I (data not shown), a null strain with a deficiency at position −392 to −486 (Muskavitch and Hogness, 1982). These results suggest that DNA sequences corresponding to the above deletions may participate in the mechanism underlying enhancement of Sgs-4 expression.

Table 1. Enhancement of Weakly Expressed Alleles in Various Heterozygotes

Male _____ Female	Ber-I (0%)	Hikone-R (~2%)	Seto (~2%)	Canton-S (40%–50%)	D323 (40%–50%)	Oregon-R (100%)	Confluens (200%)
Kochi	~1	~1	~1	4.98 ± 1.32	4.90 ± 1.28	4.46 ± 1.76	9.20 ± 3.20
Hikone-R	–	–	–	–	–	~1	2.12 ± 0.28
Seto	–	–	–	–	–	1.30 ± 0.26	~1
Ber-I	–	–	–	–	–	~1	~1

The top row indicates strains from which males were obtained. Tester females were obtained from weakly expressed strains listed in the leftmost column. The percentages indicate the normal level of an Sgs-4 allele's expression relative to Oregon-R (100%). Table entries were derived by the following formula:

$$\left(\frac{[Sgs\text{-}4 \text{ cpm}/Sgs\text{-}5 \text{ cpm}]_{heterozygote}}{[Sgs\text{-}4 \text{ cpm}/Sgs\text{-}5 \text{ cpm}]_{homozygous\ control}} \right) \times 2$$

where homozygous control indicates the strain from which the tested female was obtained. Each entry was multiplied by a factor of 2 since two copies of the tested Sgs-4 allele are present in the homozygote whereas only a single copy is present in the heterozygote. Experimental cpm values were obtained by sequential hybridization of a Northern blot with probes for Sgs-4, Sgs-5, and rRNA. Each RNA was cut out of the filter and quantitated by scintillation spectrometry. Normalization of Sgs-4 cpm values by Sgs-5 and rRNA cpm values yielded similar results. Hence we confine our analysis to the Sgs-4/Sgs-5 ratio. The standard deviations shown represent counting errors associated with scintillation spectrometry, transformed by the arithmetical manipulation described in the above formula. All experiments were repeated at least once. A value of ~1 indicates there was no expression enhancement. Quantitation was usually not possible in these instances, either because there was no detectable RNA or because it was in such low abundance as to be essentially at background cpm levels. Dashes indicate heterozygotes that were not tested.
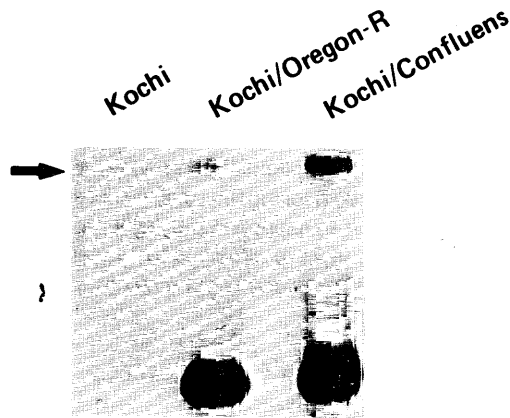
Figure 2. Enhancement of Kochi *Sgs-4* Expression Is Proportional to the Dose of Strongly Expressed *Sgs-4* Allele

This Northern blot was prepared and hybridized with Dm1513 as described in Figure 1. The arrow indicates the Kochi *Sgs-4* RNA common to all lanes. The remaining RNAs are derived from the strongly expressed *Sgs-4* alleles used in the screen.
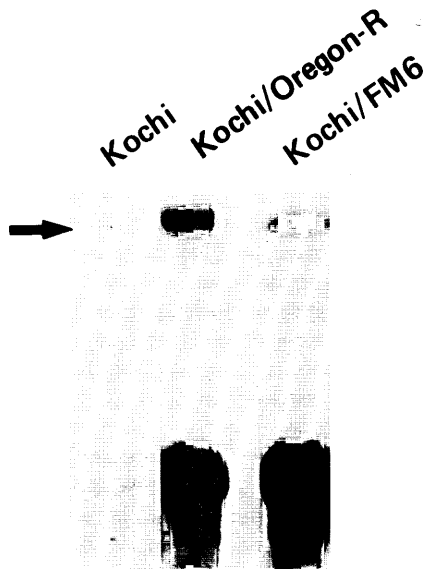

Figure 3. Disruption of Somatic Pairing Reduces Enhancement of Kochi *Sgs-4* Expression

Northern preparation and hybridization with Dm1513 have been described. The arrow indicates the Kochi *Sgs-4* RNA common to all lanes. The remaining RNAs are derived from the strongly expressed *Sgs-4* alleles used in the screen.

Gross Disruption of Somatic Pairing at 3C Greatly Reduces the *Sgs-4* Enhancement Effect

To determine if the enhancement effect is dependent upon pairing of chromosome homologs, a *Kochi/In(1)FM6* heterozygote was constructed. *In(1)FM6* is an X chromosome inversion with a break point in the 3C region and bears an *Sgs-4* allele that is expressed to ~75% of *Sgs-4*^{ORE} levels. Pairing disruption at 3C in *Kochi/FM6* heterozygotes occurred in 79% of the nuclei examined (n = 58). The *In(1)FM6* chromosome effectively disrupts pairing at
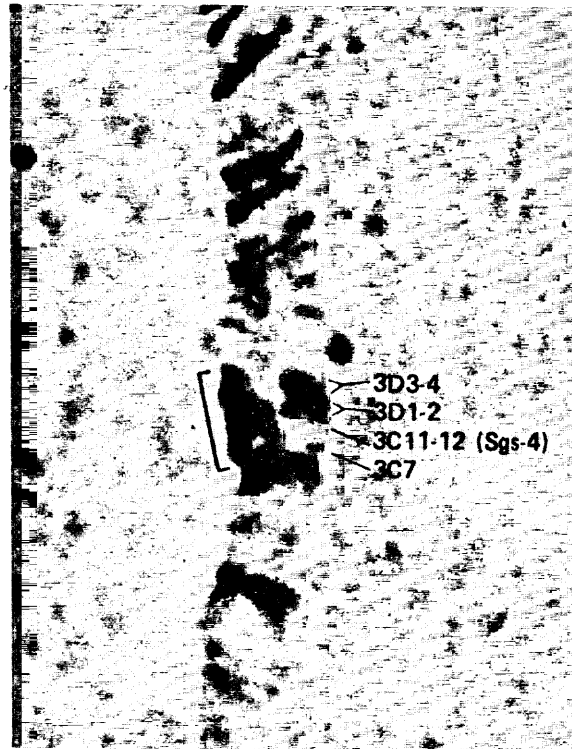

Figure 4. The 3C Region Is Asynapsed in *Kochi/Confluens* Heterozygotes.

The band designation demarcate the 3C and 3D regions of the Kochi X chromosome. The bracket indicates the portion of the *Confluens* X chromosome that contains the tandem duplication. The phase dark cytology of this duplication is typical and has been noted previously (Morgan et al., 1941).

the *Sgs-4* locus. *Sgs-4*^{K} expression in *Kochi/FM6* heterozygotes is enhanced ~1.5-fold over that in Kochi females (see Figure 3). This value is consistent with the enhancement expected from a *Kochi/Oregon-R* heterozygote, in which only 21% of the nuclei are subject to expression enhancement. Similar results were obtained using another X chromosome inversion, *In(1)FM4*. These results indicate that gross disruption of somatic pairing in the neighborhood of the *Sgs-4* locus can greatly reduce the enhancement effect.

Pairing Disruption at 3C Such That Proximity of Homologs Is Maintained Is Associated with Maximal Kochi *Sgs-4* Enhancement

When the polytene chromosomes of larval salivary glands from *Kochi/Confluens* heterozygotes are fixed, stained with lactic acetic orcein (LAO), and examined with a phase contrast microscope we find that pairing is disrupted in the 3C region (see Figure 4). It is difficult to ascertain the percentage of nuclei in which the 3C region is unpaired because the tandem elements of the duplication tend to associate with each other (Morgan et al., 1941), thereby obscuring the region's cytology. However, for the nuclei that can be examined, there is no evidence of complete pairing within the 3C region. This observation is not unexpected given that there is a duplication of 13 to 15 polytene

bands in the 3C region of the *Confluens* chromosome. Pairing of this additional DNA with the single 3C region on the Kochi-derived homolog could introduce such local torsional stress as to render complete synapsis impossible.

Discussion

Korge (1977, 1981) has shown enhanced accumulation of the Sgs-4^K protein in the salivary gland lumen of larvae heterozygous for Kochi and Oregon-R. He observed similar enhancement of Sgs-4 protein when either Samarkand or Hikone-R was made heterozygous with Oregon-R. We have shown substantial enhancement of Sgs-4^K expression at the level of transcription. We have also found marginal enhancement of Sgs-4^{HIK} expression at the level of transcription. But we find no Oregon-R-associated enhancement of Sgs-4^{HIK} transcript levels and, at best, a marginal enhancement effect on Samarkand expression (data not shown).

Expression Enhancement and the Role of Transcriptional Factors
The enhancement of Sgs-4^K expression when juxtaposed with *Confluens* or Oregon-R could be explained in terms of varying levels of Sgs-4 transcriptional factors. The introduction of a strongly expressed Sgs-4 gene into a Kochi strain may result in the sequestering of greater numbers of some limiting transcriptional factors at the 3C region than occurs in the Kochi homozygote. The extent of this enhancement would then be expected to be proportional to the concentration of transcriptional factors in the 3C neighborhood. The introduction of two strongly expressed Sgs-4 genes (e.g. *Confluens*) increases the titer of transcriptional factors proportionately, with a corresponding increase in induced Sgs-4^K transcription. Weakly expressed Sgs-4 alleles will not increase the local density of transcriptional factors and thus fail to produce enhanced expression as we have observed. These limiting transcriptional factors could act either at the 5' end of the gene to stimulate initiation of transcription or on the gene itself during the transcription process. This interpretation assumes that our measure of accumulated Sgs-4 transcript accurately reflects transcriptional activity at the Sgs-4 locus.

The observation that Sgs-4^K expression is subject to the same levels of enhancement by Oregon-R and by strains with Sgs-4 expression at 40% to 50% that of Oregon-R (D323, Canton-S) suggests that there may be a threshold concentration of transcriptional factors which must be attained before additional expression can be induced.

Specific Upstream Sequences Are Necessary for the Enhancement Effect
We have evidence that specific 5' flanking sequences are necessary to ensure expression enhancement. In the *Confluens/Ber-I* heterozygote we find no Sgs-4^{BER} transcript. The failure to induce Ber-I expression might be accounted for by the -392 to -486 deficiency associated

with the Sgs-4^{BER} gene and implicated in its null expression (Muskavitch and Hogness, 1982). This region may be required for the binding of transcriptional factors. It is noteworthy that Shermoen and Beckendorf (1982) have mapped a major DNAase I hypersensitive site to this deficiency. Whether transcriptional factors associate directly with the 5' sequences flanking the weakly expressed Sgs-4 allele or influence transcription propagation, the presence of increasing titers of diffusible transcriptional factors suggests that the enhancement effect may act in cis as well as in trans.

Similarities between Sgs-4 Expression Enhancement and Transvection
Proximity-induced enhancement of Sgs-4 expression is similar in several ways to the phenomenon of transvection first observed by Lewis in *bithorax* (1954). Transvection, simply stated, is an alteration in allelic complementation arising from the pairing-dependent interaction of alleles. In general, if pairing is disrupted at the site of the allelic interaction, then this complementation is decreased (Lewis, 1954; Jack and Judd, 1979; Gelbart, 1982).

The minimal enhancement of Kochi expression observed in *Kochi/FM6* heterozygotes is similar to the disruption of genetic complementation by chromosomal rearrangement observed in loci subject to transvection. The presence of some enhancement is not surprising given that the In(1)FM6 rearrangement does not completely disrupt somatic pairing at 3C. Sgs-4^{FM6} expression is sufficient to induce Sgs-4^K expression if the alleles remain paired or in proximity. Nevertheless, the substantial reduction in Sgs-4^K enhancement observed in *Kochi/FM4* and *Kochi/FM6* heterozygotes suggests that enhanced Sgs-4 expression is sensitive to gross pairing disruptions and thus its behavior is consistent with that expected of a gene subject to transvection.

Microenvironments and the Enhancement Effect
The cytological evidence that Sgs-4^K and Sgs-4^{Co} alleles fail to pair at 3C and yet result in the most pronounced enhancement effect observed encourages us to suggest the following working hypothesis. Transcriptional enhancement of Sgs-4 is mediated by diffusible transcriptional factors. These factors are sequestered by specific upstream sequences, e.g. enhancer-like domains, creating a local increase in their concentration. Paired homologous genes within this domain can utilize these factors to promote their transcription. The greater the concentration of available transcriptional factors, arising for example by the introduction of additional upstream sequences, the greater the likelihood for utilization of these factors by the homologous gene. If the homologous gene is removed from this domain by chromosomal rearrangement or if it lacks the proper factor recognition sequences, then its expression will not be influenced by these factors. Given that precise pairing of homologs at 3C is not necessary for this effect (as evidenced in *Kochi/Confluens* heterozygotes) we suggest that proximity of homologous loci may be sufficient to produce enhancement of a weak Sgs-4 allele's expression. Proximity-dependent enhancement may apply to in-

teractions between strongly expressed *Sgs-4* alleles. However, this could be difficult to establish with alleles producing a high background level of expression. If this is the case, then to establish a more general case for expression enhancement at other loci may require a collection of hypomorphic alleles.

We are currently mapping the enhancement effect to the *Sgs-4* locus and utilizing P element mediated germ line transformation to delineate the upstream sequences associated with expression enhancement.

Experimental Procedures

Description of Drosophila Stocks
Flies were raised at 27°C on standard fly food consisting of cornmeal, molasses, and brewer's yeast. The Ber-I and S8D strains were provided by Steve Beckendorf. The Canton-S and Oregon-R (Stanford) strains were provided by Ken Burtis. The Samarkand, D323, Kochi, Seto, and Hikone-R strains as well as the *In(1)FM6* and *In(1)FM4* stocks were obtained from the Drosophila Stock Center at Bowling Green, Ohio.

Plasmids
Dm1513, kindly provided by Marc Muskavitch, is an EcoRI subclone of Dm1528 that contains all 900 bp of the Oregon-R *Sgs-4* reading frame plus 900 bp of downstream flanking DNA inserted into the EcoRI site of pBR325 (M.A.T. Muskavitch, Ph.D. thesis, Stanford University, 1981).

Dm1687, courtesy of Greg Guild, is a HindIII subclone of Dm1508 that contains all 750 bp of the Oregon-R *Sgs-5* reading frame inserted into the HindIII site of pBR322 (Guild, 1984).

Dm103, provided by Ken Burtis, is a 17 kb EcoRI fragment of rDNA containing the long 18S and 28S repeat inserted into the EcoRI site of pSC101 (Glover and Hogness, 1977).

Purification of Nucleic Acids
Plasmid DNA was isolated according to standard procedures (Maniatis et al., 1982). RNA from third instar larval salivary glands was isolated according to Stellar and Pirotta (1984).

RNA Electrophoresis and Northern Blotting
RNA was glyoxylated according to the method of McMaster and Carmichael (1977). Glyoxylated RNA was electrophoresed through neutral agarose gels and blotted onto nitrocellulose according to Thomas (1980). Northern blots were then baked and incubated at 42°C in the following buffer during prehybridization and hybridization: 50% formamide, 5× SSC, 50 mM NaPO$_4$ (pH 6.5), 7.5× Denhardt's, 10 mg salmon sperm DNA. Plasmid DNA was labeled in vitro by nick translation utilizing ^{32}P-dCTP (3000 Ci/mmol, Amersham).

Quantitation of RNAs
A nitrocellulose filter containing the RNAs to be quantitated was branded with radioactive ink and exposed to X-ray film in the presence of a Cronex intensifying screen at −80°C. The filter and its corresponding autoradiogram were aligned; those segments of filter bearing the appropriate RNAs were cut out, placed in scintillation cocktail (ACSII, Amersham), and subjected to liquid scintillation spectrometry. Several sectors of nitrocellulose not hybridized by the probe were also selected for a background cpm value.

Fixing and Staining Larval Salivary Glands
Larval salivary glands were fixed and stained with 2% lactic-acetoorcein as described by Sharma and Sharma (1965). Salivary gland preparations were examined with phase contrast optics utilizing a 100× planapochromat oil immersion lens (numerical aperture 1.3).

References

Ashburner, M. (1970). The genetic analysis of puffing in polytene chromosomes of *Drosophila*. Proc. Royal Soc. Lond. B. *176*, 319–327.

Gelbart, W. M. (1982). Synapsis-dependent allelic complementation at the decapentaplegic gene complex in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *79*, 2636–2640.

Glover, D. M., and Hogness, D. S. (1977). A novel arrangement of the 18S and 28S sequences in a repeating unit of Drosophila melanogaster rDNA. Cell *10*, 167–176.

Guild, G. M. (1984). Molecular analysis of a developmentally regulated gene which is expressed in the larval salivary gland of *Drosophila*. Dev. Biol. *102*, 462–470.

Jack, J. W., and Judd, B. H. (1979). Allelic pairing and gene regulation: a model for the zeste-white interaction in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *76*, 1368–1372.

Korge, G. (1977). Direct correlation between a chromosome puff and the synthesis of a larval saliva protein in *Drosophila melanogaster*. Chromosoma *62*, 155–174.

Korge, G. (1981). Genetic analysis of the larval secretion gene *Sgs-4* and its regulatory chromosome sites in *Drosophila melanogaster*. Chromosoma *84*, 373–390.

Lewis, E. B. (1954). The theory and application of a new method of detecting chromosomal rearrangements in *Drosophila melanogaster*. The American Naturalist *88*, 225–239.

Lindsley, D., and Grell, E. H. (1968). Genetic variations of *Drosophila melanogaster*. Carnegie Inst. Wash. Publ. No. 627.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning. A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

McMaster, G. K., and Carmichael, G. G. (1977). Analysis of single and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA *74*, 4835–4838.

Metz, C. W. (1916). Chromosome studies on the diptera II. The paired association of chromosomes in the Diptera, and its significance. J. Exp. Zool. *21*, 213–279.

Morgan, T. H., Schultz, J., and Curry, V. (1941). Investigations on the constitution of the germinal material in relation to heredity. Carnegie Inst. Wash. Year Book *40*, 282–287.

Muskavitch, M. A. T., and Hogness, D. S. (1980). Molecular analysis of a gene in a developmentally regulated puff of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *77*, 7362–7366.

Muskavitch, M. A. T., and Hogness, D. S. (1982). An expandable gene that encodes a Drosophila glue protein is not expressed in variants lacking remote upstream sequences. Cell *29*, 1041–1051.

Sharma, A. K., and Sharma, A. S. (1965). Chromosome Techniques, Theory and Practice. (Washington, D.C.: Butterworths), 474 pp.

Shermoen, A. W., and Beckendorf, S. K. (1982). A Complex of Interacting DNase I-Hypersensitive Sites near the Drosophila Glue Protein Gene, *Sgs-4*. Cell *29*, 601–607.

Stellar, H., and Pirotta, V. (1984). Regulated expression of genes injected into early *Drosophila* embryos. EMBO J. *3*, 165–173.

Thomas, P. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA *77*, 5201–5205.