STUDIES ON THE MECHANISM OF HETEROCHROMATIC POSITION EFFECT AT THE ROSY LOCUS OF *DROSOPHILA MELANOGASTER*

C. A. RUSHLOW,*[,1] W. BENDER† AND A. CHOVNICK*[,2]

Molecular Genetics and Cell Biology Section, Biological Sciences Group, The University of Connecticut, Storrs, Connecticut 06268; and †Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetss 02115

Manuscript received February 15, 1984
Revised copy accepted June 14, 1984

ABSTRACT

Experiments are described that extend the characterization of position effect variants of the rosy locus and test possible mechanisms of heterochromatic position effect.——Rosy position effect variants exhibit a variegated phenotype with respect to xanthine dehydrogenase activity in malpighian tubules.——The breakpoints of the position effect mutations are located on the DNA map of the rosy region outside of the rosy locus DNA; $ry^{ps11136}$ is located in the DNA of the *l(3)S12* gene immediately proximal to rosy, whereas ry^{ps1149} is located some 15 kb distal to rosy in the *pic* locus.——Southern blot experiments are described that test and reject the notion that heterochromatic position effect results from underreplication of the position-affected gene. Rather, the results of Northern blots serve to direct attention to position effect as a defect in transcription.——Histone region deletion heterozygosity and butyrate-feeding experiments failed to exhibit specific suppression of position effect at the rosy locus.

THE accompanying report (RUSHLOW and CHOVNICK 1984) describes observations and experiments utilizing classical genetic and biochemical techniques to characterize two radiation-induced "leaky" mutants at the rosy locus, ry^{ps1149} and $ry^{ps11136}$, as position effect mutants associated with heterochromatic rearrangements. The present report represents a continued characterization of these position effect variants with particular emphasis on molecular characterization and tests of possible mechanisms of position effect.

MATERIALS AND METHODS

Mutants, special chromosomes, rearrangements and balancers: See the accompanying report (RUSHLOW and CHOVNICK 1984).

The 79B actin gene probe was kindly provided by ERIC FYRBERG of John Hopkins University.

DNA extraction: Whole animals or dissected tissues were homogenized in 0.2 M sucrose, 0.1 M NaCl, 0.05 M EDTA, 0.5% SDS, 0.1 M Tris, pH 9.1. After at least five min, potassium acetate was added to a final concentration of 1 M, and the mixture was set on ice for 1 hr. The homogenate was centrifuged at 10,000 rpm for 5 min. An equal volume of 100% ethanol was added to the supernatant,

[1] Present address: ICRF, Burtonhole Lane, London NW7 1AD, England.
[2] To whom reprints requests should be addressed.

and after 5 min at room temperature, the mixture was centrifuged for 5 min at 10,000 rpm. The pellet was dried and resuspended in TE (0.01 M Tris, pH 8.0, 0.001 M EDTA).

DNA quantitation: Standards for DNA measurements were prepared by dissolving 100 mg of calf thymus DNA (Sigma) in 100 ml of 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and centrifuging for 30 min at 40,000 rpm in a Beckman Ti50 rotor. The supernatant was brought to 0.25 M NaCl, and the DNA was precipitated by the addition of 2 volumes of 95% ethanol. The DNA was resuspended in 0.1 × SSC and the DNA content was determined in a Beckman model 25 spectrophotometer (1 OD = 41.5 μg/ml).

Aliquots of the DNA samples or DNA standards were diluted into distilled water to a final volume of 0.3 ml/tube. One hundred microliters of 2 M diaminobenzoic acid (DABA) (Sigma), prepared as described by KISSANE and ROBINS (1958), were added to each tube, and the samples were incubated at 60° for 90 min. The reaction was terminated by the addition of 2.5 ml of 0.6 N perchloric acid, and the samples were read in a Perkin Elmer fluorometer with exciter and analyzer wavelength of 408 and 502 nm, respectively.

RNA extraction: All water and buffers were filtered three times through nitrocellulose type HA 0.45-μm filters. All glassware was baked for at least 6 hr at 200°. Larvae (3–4 g of crawling third instar) or adults (3-4 g up to 2 days old) were frozen at −80° prior to use. Each sample was homogenized in 10 ml of homogenization buffer (0.1 M sodium acetate, pH 5.5, 0.5% Sarkosyl) for 30 sec in a Sorvall Omnimixer. An equal volume of hot phenol (60°) saturated in buffer was added and the mixture homogenized for an additional 3 min. The phenol phase was reextracted, and the collected aqueous phase was extracted twice more. Sodium acetate was added to a final concentration of 0.5 M and the nucleic acid precipitated upon addition of 3 volumes of 100% ethanol and stored at −20°. Quantitation is by UV spectrophotometry.

Oligo-dT chromatography: To separate polyadenylated RNA from nonpolyadenylated RNA, oligo-dT chromatography was employed (AVIV and LEDER 1972). The powder was soaked in 0.1 M NaOH for at least 1 hr prior to use as were the Bio-Rad disposo-columns. The slurry was poured into the columns and equilibrated with binding buffer (0.5 M NaCl, 0.02 M Tris, pH 7.5). Samples were resuspended in 3 ml of water, heated for 5 min at 65°, chilled in ice water and brought to binding buffer salt concentrations. The samples were applied four times to the column, and the column was rinsed with 20 ml of binding buffer. Poly-A^+ RNA was eluted with water and precipitated in 0.5 M NaCl and 75% ethanol at −20°. Samples were resuspended in small volumes of water.

Restriction enzyme digests: Restriction enzymes were obtained from Bethesda Research Laboratories, Inc. DNA was incubated with enzyme following the manufacturer's suggested protocol. Reactions were stopped by the addition of ⅕ volume of the solution; 0.1 M EDTA, 1 mM Tris, pH 7.5, 1.5% SDS, 0.1% bromphenol blue and 2% Ficoll. Samples were then applied to 0.9% agarose gels.

Agarose gel electrophoresis, nondenaturing: Agarose gels, 0.9% were prepared and run in E buffer (0.04 M Tris, pH 8.3, 0.02 M sodium acetate, 0.02 M EDTA) until the tracking dye (bromphenol blue) had migrated approximately 13 cm.

Agarose gel electrophoresis, denaturing: Agarose gels, 1%, containing 2.2 M formaldehyde (37%) were prepared and run in RNA gel buffer (0.2 M Hepes or MOPS, pH 7.0, 0.05 M sodium acetate, 0.001 M EDTA) (B. SEED and D. GOLDBERG, unpublished results). Samples containing RNA resuspended in water, 1X gel buffer, 50% formamide and 2.2 M formaldehyde were heated at 65° for 5 min and chilled in ice water. The gel was run at 150 V until the tracking dye had migrated approximately 13 cm.

Gel blots: Transfer of DNA or RNA fragments from the agarose gel to nitrocellulose (Schleicher and Schuell) was achieved by the technique of SOUTHERN (1975). Following DNA gel electrophoresis and ethidium bromide staining, the gel was soaked in 1.5 M NaCl, 0.5 M NaOH for 30 min, rinsed and soaked in 1.0 M Tris, pH 8.0, 1.5 M NaCl. The gel was blotted against nitrocellulose filter paper with 20 × SSPE (SSPE = 3.0 M NaCl, 0.2 M $NaHPO_4$, pH 7.0, 0.04 M EDTA) THOMAS (1980). Following RNA gel electrophoresis, the gel was blotted against nitrocellulose filter paper, and filters were baked in a vacuum oven for 2 hr at 80°.

Filter hybridization: ^{32}P-labeled DNA (specific activity 3-4 × 10^8 cpm/μg) was prepared by the method of RIGBY *et al.* (1977). Probes were prepared as follows: 5 ml of formamide (Fluka), 2.5 ml of 20 × SSPE, 0.2 ml of Denhardt's solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinyl pyrrolidone) and 0.01 ml 10% SDS were mixed with 1 g of Dextran sulfate (500,000 daltons). This mixture was

added to the DNA (1 ml of ^{32}P-labeled DNA in TE and 1 ml of salmon sperm competitor DNA), which was heated (95°) for 3-5 min and cooled in ice water. This probe was prehybridized with nitrocellulose for at least 3 hr at 42°. It was again denatured by heating, cooled and hybridized to blotted filters for at least 10 hr at 42°. Following hybridization, filters were washed six times in decreasing salt concentrations, each time for 30 min at 42°: (1) Formamide (50%), 5 × SSPE, 1 × Denhardt's solution, 0.1% SDS, (2) 2 × SSPE, 1 × Denhardt's, 0.1% SDS, (3) and (4) 2 × SSPE, 0.1% SDS, (5) and (6) 0.1 × SSPE, 0.1% SDS. The filters were damp dried and wrapped in plastic wrap but not allowed to air dry. They were then exposed to X-ray film (Kodak) at −80°.

Malpighian tubule histochemistry: Late third instar larvae were dissected in 0.2 M Tris (pH 8.2). Malpighian tubules were incubated in a staining solution (0.5 mg/ml of hypoxanthine, 1.0 mg/ml of nitroblue tetrazolium, 0.3 mg/ml of phenazine methosulfate and 0.1 mg/ml of NAD in 0.2 M Tris at pH 8.2) for 5 min at 55°. The tubules were rinsed in cold buffer, examined and stored at 4° in the dark. To obtain uniform staining of tubules of ry^+ larvae, and repeatable results with ry^+ and position-affected genotypes, care was taken not to damage the tissue during dissection. Additionally, constant temperature during the staining reaction was essential.

RESULTS

Position-affected rosy locus expression in larval malpighian tubules: In the accompanying report (RUSHLOW and CHOVNICK 1984), all of the data that served to characterize position-affected rosy locus expression involved observations on nonautonomous phenotypes. Taking advantage of the pleiotropic nature of rosy locus expression, we were able to demonstrate the variegated nature of the rosy position effect in malpighian tubule cells. Figure 1 presents XDH-specific stained preparations (see MATERIALS AND METHODS) of excised malpighian tubules of ry^+, ry^- and position-affected mutant late third instar larvae of the indicated genotypes. Figure 1a illustrates the apparent uniform deposition of purple formazan granules in cells over most of the ry^+ tissue.The anterior-most section of the anterior tubules appear to be lightly stained (Figure 1a). The total absence of XDH activity in malpighian tubules of ry^- mutants is illustrated in Figure 1b. Formazan granule deposition in the ureter and gut is variable and nonspecific as seen in the ry^- and other preparations. Figure 1c and d are preparations from our position effect mutants, $ry^{ps11136}$ and ry^{ps1149}. Although variegation at the level of XDH activity appears to be consistent with a clonal pattern of "all or none" cell expression, a firm statement on this point is not yet technically feasible.

Location of the ry^{ps1149} *and* $ry^{ps11136}$ *rearrangement breakpoints on the restriction map of cloned rosy region DNA*: The DNA of the rosy region has been cloned by genomic walking (BENDER, SPIERER and HOGNESS 1983), and lesions associated with several rosy mutants are clustered within the 5-kb region noted in Figure 2 (B. COTE, W. BENDER and A. CHOVNICK, unpublished results). This localization of the rosy DNA has been confirmed by transformation studies; both the 8.1-kb *Sal*I fragment and the 7.2-kb *Hin*dIII fragment (Figure 2) complement rosy mutants (SPRADLING and RUBIN 1983).

Southern blot analyses were carried out on DNAs extracted from the ry^{+11} parental strain and the position-affected mutant derivatives, ry^{ps1149} and $ry^{ps11136}$. We have argued that the closely linked lethalities associated with the position-effect rearrangement chromosomes (*pic-* with ry^{ps1149} and $l(3)S12^-$ with $ry^{ps11136}$) in fact are due to the location of the rearrangement breaks within those genes (RUSHLOW and CHOVNICK 1984). If true, then localization of the breakpoints on

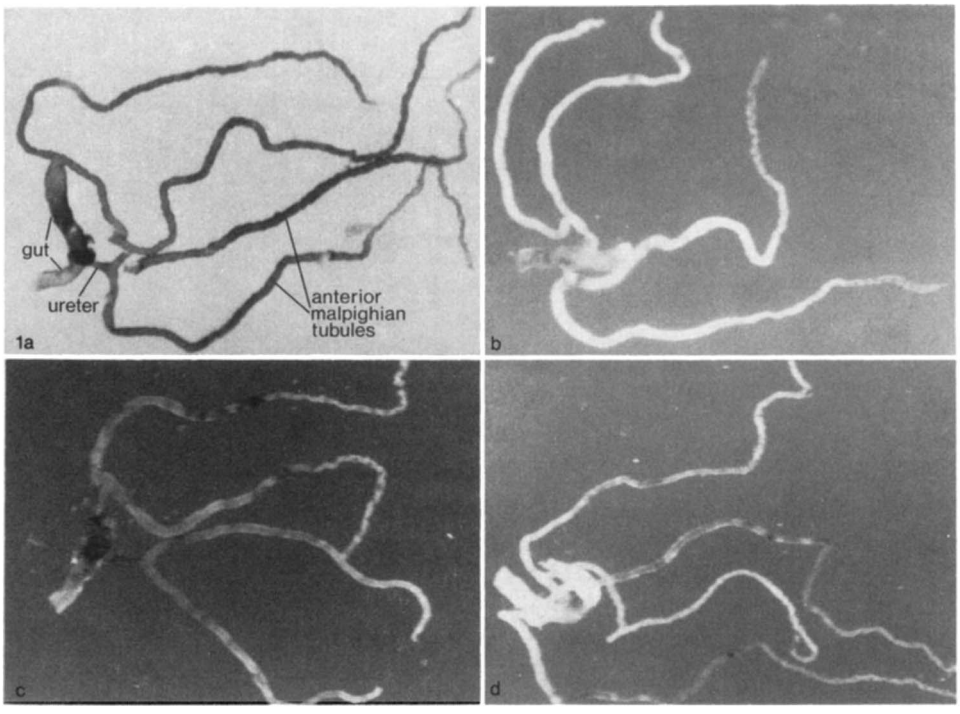

FIGURE 1.—Larval malpighian tubules (late third instar females) of (a) $ry^{+11}/MKRS$, (b) ry^{506}/ry^{506}, (c) $ry^{ps11136}/MKRS$ and (d) $ry^{ps1149}/MKRS$.

the DNA map would be evident from comparison of restriction patterns of the parental ry^{+11} DNA and position effect DNA probed with cloned DNA segments which span the region flanking rosy (phage clones 2840, 2833 and 2827 of Figure 2). The results, summarized in Figure 2, locate the rearrangement breakpoints quite precisely. The $ry^{ps11136}$ breakpoint is located between the left *Sal*I and the left *Hin*dIII sites that mark the left ends of the 8.1-kb *Sal*I and 7.2-kb *Hin*dIII rosy locus fragments (Figure 2). The ry^{ps1149} breakpoint is located some 15 kb distal to the rosy locus (Figure 2). That these breakpoints, in fact, mark the DNA of the *l(3)S12* and *pic* genes, respectively, is supported by the following additional facts. (1) The lethalities associated with these position effect rearrangement chromosomes are not part of a pattern of radiating position effects, and the lethalities are not *Y* suppressed (RUSHLOW and CHOVNICK 1984). (2) Transformation with the 8.1-kb *Sal*I fragment complements *l(3)S12* as well as rosy, whereas the 7.2-kb *Hin*dIII transformant only complements rosy locus function (S. CLARK and A. CHOVNICK, unpublished observations). (3) Several additional chromosome *3* rearrangements that are *pic* lethal have DNA breaks very close to that of ry^{ps1149} (SCOTT *et al.* 1983; B. COTE, W. BENDER and A. CHOVNICK, unpublished results).

Comparison of rosy DNA template levels in ry^{+11} *and* $ry^{ps11136}$ *fat bodies*: Since function of the rosy locus is principally in the polytene fat body and malpighian tubule tissues (See discussion in RUSHLOW and CHOVNICK 1984), and since

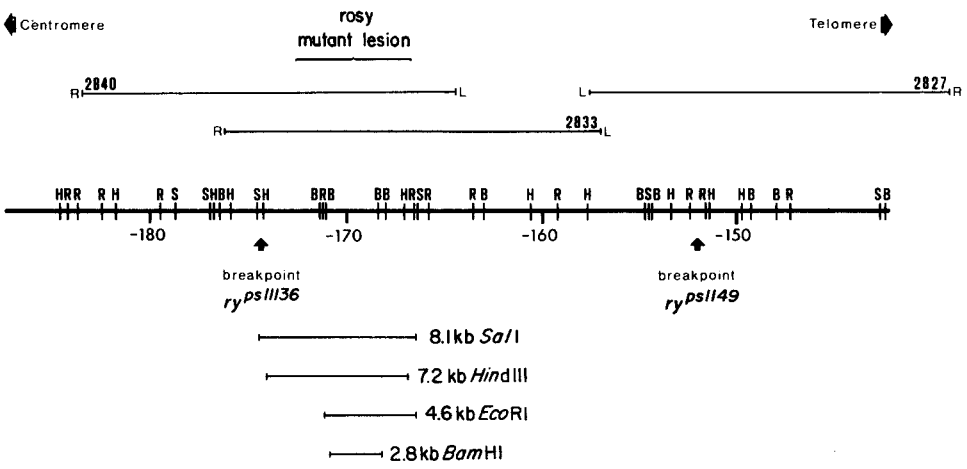

FIGURE 2.—Restriction map of rosy region DNA. The bold-faced line represents the map from the Canton S wild type for the four restriction enzymes *Bam*HI, *Hin*dIII, *Eco*RI and *Sal*I (B, H, R and S, respectively). The coordinates show distance in kilobases from the start point of the walk (BENDER, SPIERER and HOGNESS 1983). The lines above the map indicate the cloned DNA segments from the Canton S library of recombinant phage. The L and R designations indicate the orientation of the left and right arms of the lambda vector. The lines below the map indicate the extent of particular restriction fragments. The arrows at the bottom of the figure locate the rearrangement breakpoints for the position-affected variants.

heterochromatin is relatively underreplicated in polytene tissue (RUDKIN 1965; GALL, COHEN and POLAN 1971), then the apparent quantitative reduction in XDH production and other associated mutant phenotypic effects might be due to a reduced relative amount of rosy locus DNA. Essentially, this hypothesis argues that, in the polytene fat body or malpighian tubules, the specific rosy locus DNA templates on the position effect rearrangement chromosome are relatively underrepresented in DNA extracts of these tissues, as compared with nonposition-affected rosy locus DNA.

Examination of this question involved gel blot analysis following restriction enzyme digestion of DNA extracts and electrophoresis of the restricted DNA (SOUTHERN 1975). *Hin*dIII-digested ry^{+11} DNA will produce a 7.2-kb fragment (Figure 2). In contrast, the *MKRS* balancer chromosome, marked by the mutant, ry^{2}, produces an 11.5-kb *Hin*dIII fragment. This is due to the fact that the ry^{2} mutation is associated with an insertion of the mobile element, *B104* (B. COTE, W. BENDER and A. CHOVNICK, unpublished results), which was originally characterized by SCHERER *et al.* (1982). Consequently, gel blots of *Hin*dIII-digested DNA from the heterozygote, $ry^{+11}/MKRS$ or $ry^{ps1112^{36}}/MKRS$, should reveal two rosy specific bands (7.2 and 11.5 kb) upon exposure to an appropriate rosy locus DNA probe. Figure 3 illustrates an autoradiogram of such a gel blot that has been probed with ^{32}P-labeled plasmid (pBR322) DNA containing the 4.6-kb *Eco*RI fragment (Figure 2). Lanes 1 through 4 represent a series of doubling dilutions of DNA extracted from $ry^{+11}/MKRS$ adults. The diminution of intensity of the bands from lanes 1 to 4 are consistent with the doubling dilutions and indicate that the film is not under- or overexposed. Lanes 5 and 6 contain DNA

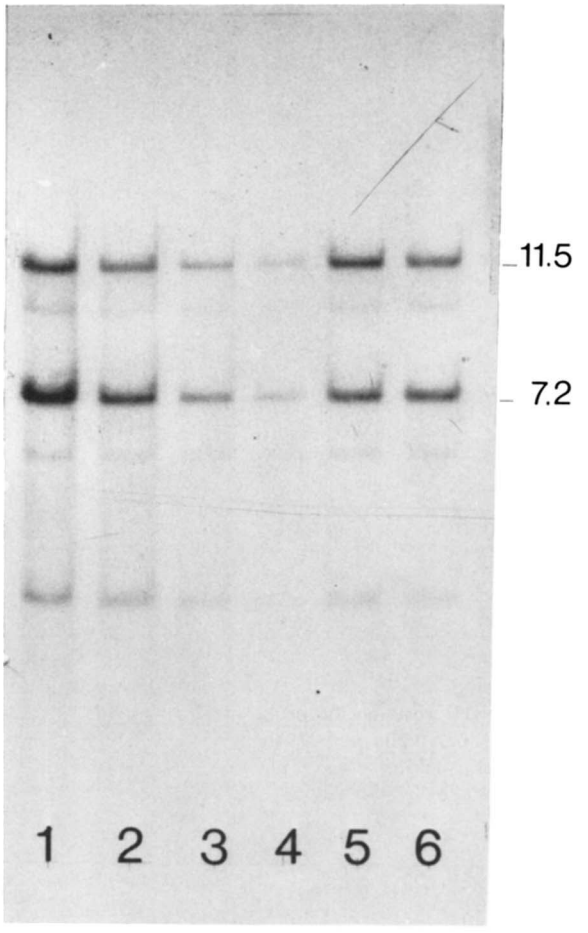

FIGURE 3.—Whole genome Southern blot autoradiogram. The blot was probed with ^{32}P-labeled rosy-specific DNA (4.6 kb *Eco*RI). DNA applied to lanes 1 through 6 in the following amounts and from the indicated sources: (1) 4 μg ry^{+11}/*MKRS* adult female, (2) 2 μg ry^{+11}/*MKRS* adult female, (3) 1 μg ry^{+11}/*MKRS* adult female, (4) 0.5 μg ry^{+11}/*MKRS* adult female, (5) 2 μg $ry^{ps11136}$/*MKRS* late third instar female larval fat body, (6) 2 μg ry^{+11}/*MKRS* late third instar female larval fat body.

extracted from isolated larval fat body tissue of $ry^{ps11136}$/*MKRS* and ry^{+11}/*MKRS*, respectively. XDH levels in isolated larval fat bodies exhibit the position effect reduction comparable to that of whole larvae and adults (RUSHLOW and CHOVNICK 1984). There is no apparent difference between the 7.2-kb bands in lanes 5 and 6 as would be expected if the position-affected rosy DNA templates were underrepresented in the extract. One less DNA replication of the position-affected gene would have produced a 50% reduction in the 7.2-kb band of lane 5 as compared with that of lane 6.

An even more dramatic result is seen in Figure 4 which illustrates an experiment that was carried out to examine the effect of variation in *Y* chromosome heterochromatin on position-affected and ry^+-specific template content in DNA extracts of larval fat bodies. Since the most extreme position effect is seen in *XO*

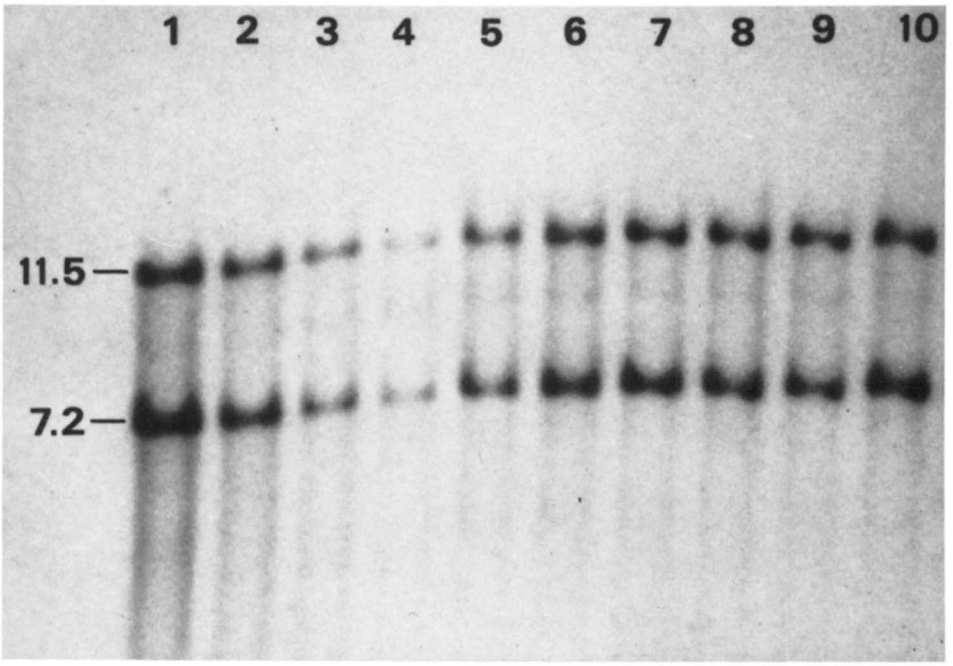

FIGURE 4.—Whole genome Southern blot autoradiogram. DNA was digested with *Hin*dIII and hybridized with a 2.7-kb *Bam*HI rosy-specific probe (internal to the 4.6-kb *Eco*RI fragment, see Figure 2). Lanes 1–4 contain 1, 0.5, 0.25 and 0.175 μg of ry^{+11}/*MKRS* adult female DNA, respectively. Lanes 5–10 contain 0.5 μg of third instar larval fat body DNA as follows: (5) ry^{+11}/*MKRS XX*, (6) ry^{+11}/*MKRS XY*, (7) $ry^{ps11136}$/*MKRS XX*, (8) $ry^{ps11136}$/*MKRS XY*, (9) $ry^{ps11136}$/*MKRS XXY*, (10) $ry^{ps11136}$/*MKRS XO*.

males, it is instructive to compare, in particular, lanes 6, 8 and 10 of Figure 4 which contain DNA from ry^{+11}/*MKRS, XY* males; $ry^{ps11136}$/*MKRS, XY* males; and $ry^{ps11136}$/*MKRS XO* males. These genotypes exhibit a sevenfold range of XDH activity differences (RUSHLOW and CHOVNICK 1984) yet show no evidence of quantitative differences in rosy-specific templates.

Similar Southern blot experiments were carried out on DNA extracts of excised malpighian tubule and salivary gland tissues with the identical result. Consequently, we reject the hypothesis that the rosy position effect involves an underrepresentation of rosy locus DNA in the polytene cells that are the principal sources of XDH.

Comparison of rosy specific poly-A$^+$ RNA transcript levels associated with ry^{+11} *and* $ry^{ps11136}$: Here, we consider the hypothesis that the position effect operates at the level of transcription and leads to the production of reduced quantities of RNA transcripts. Comparison of rosy-specific poly-A$^+$ RNA transcripts present in extracts of normal and mutant genotypes was accomplished by the Northern gel-blotting technique. To visualize and compare the transcripts produced in ry^{+11} and $ry^{ps11136}$-bearing genotypes, it was necessary to find a rosy allele that was viable in heterozyotes over $ry^{ps11136}$ and whose poly-A$^+$ RNA could be distinguished from that of ry^{+11} and $ry^{ps11136}$. Unfortunately, the ry^2 allele, present on

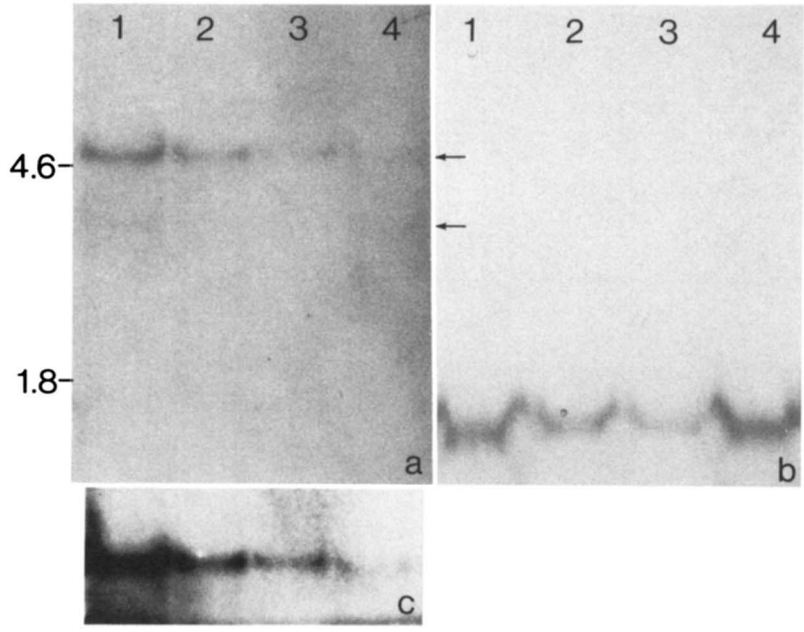

FIGURE 5.—Northern blot autoradiogram. a represents an RNA blot probed with ^{32}P-labeled rosy-specific DNA (4.6-kb *Eco*RI) and exposed for 10 days. b represents the same blot reprobed with a ^{32}P-labeled adult actin (79B) probe exposed for 3 hr. c represents the same blot probed with the ^{32}P-labeled rosy-specific DNA (4.6-kb *Eco*RI) and exposed for 3 wk. Adult female poly-A$^+$ RNA applied to each lane as indicated. (1) 30 μg ry^{+11}/ry^{506}, (2) 15 μg ry^{+11}/ry^{506}, (3) 7.5 μg ry^{+11}/ry^{506}, (4) 30 μg $ry^{ts11136}/ry^{506}$. Markers used are chick 28S rRNA ≅ 4.6 kb and chick 18S rRNA ≅ 1.8 kb.

the *MKRS* balancer chromosome, could not be used since it produces transcripts of normal size and in normal quantities. Since ry^{506} was known to be deficient for approximately 2.5 kb of the 3′ end of the rosy locus (B. COTE, W. BENDER and A. CHOVNICK, unpublished results), preliminary studies were carried out that demonstrated that poly-A$^+$ RNA extracts of ry^{506} homozygous larvae and adults possess rosy-specific transcripts of smaller than normal size (approximately 3.6 kb in contrast to 4.7 kb in wild type) and in reduced quantities.

Subsequently, crosses were carried out that generated ry^{+11}/ry^{506} and $ry^{ts11136}/ry^{506}$ adults, and their poly-A$^+$ RNAs were extracted and analyzed. Figure 5a presents an autoradiogram of a gel blot hybridized to a ^{32}P-labeled, 4.6-kb rosy locus probe and exposed to film for 10 days. The same filter was then rehybridized to a ^{32}P-labeled actin probe (adult 79B) and exposed to film for 3 hr. The 79B actin gene is associated with a 1.6-kb poly-A$^+$ RNA present in adult leg and thoracic muscle (FYRBERG *et al.* 1983). Lanes 1 through 3 of Figure 5a examine doubling dilutions of extracts of ry^{+11}/ry^{506} adults and demonstrate the separation of a 4.7-kb rosy transcript from the smaller, approximately 3.6-kb transcript of ry^{506} which is present in very much diminished quantity. Lane 4 of Figure 5a examines poly-A$^+$ RNA extracted from $ry^{ts11136}/ry^{506}$ adults at the same concentration as lane 1. There is very little of the 4.7-kb rosy-specific transcript present in this lane. That lanes 1 and 4 had similar quantities of RNA applied is seen

from the Figure 5b comparison with the actin 79B probe. Finally, the filter was exposed to film for 3 additional weeks in order to amplify whatever rosy locus probe was bound in lane 4. Prior to exposure, the filter was cut to remove the region of smaller RNAs including the heavily labeled actin 79B region (Figure 5c). Examination of Figure 5c reveals that upon greatly increasing the time for ^{32}P decay, we are able to observe the presence of a low level of rosy-specific transcript in lane 4.

From these observations, we conclude that the effect of heterochromatin placed adjacent to the rosy locus is pretranslational and most likely to be a defect in transcription leading to the production of reduced quantities of rosy locus-specific RNA transcripts. We note that the level of XDH-specific poly-A^+ RNA associated with the position effect (lane 4, Figure 5a and c) compared to the wild-type control (lane 1, Figure 5a and c) is much less than is expected by comparison in the same genotypes of enzyme activities, cross-reacting material (CRM) levels and 4-hydroxypyrazolo (3,4-d) pyrimidine (HPP) sensitivity (RUSHLOW and CHOVNICK 1984). This observation implies the existence of an additional control at the translational level.

Histones and position effect suppression: Classic notions concerning the mechanism(s) underlying position effect variegation have utilized the term "heterochromatinization" (*i.e.*, becoming like heterochromatin) to describe what might be happening to euchromatic genes brought into proximity to heterochromatin. Although this term has been used in describing the altered polytene band staining of euchromatic regions placed adjacent to heterochromatin in such rearrangements, it has also been used in relation to the associated suppression of gene functions (see reviews in LEWIS 1950; BAKER 1968; SPOFFORD 1976). It is instructive to utilize the expression heterochromatinization in pursuit of underlying mechanisms. Clearly, the facts of position effect variegation preclude hypotheses involving DNA sequence changes that might convert a euchromatic "region" into a heterochromatic one. Rather, we are drawn to notions involving DNA coiling and chromatin structural differences between functionally inactive and active DNAs (BRYAN, OLAH and BIRNSTIEL 1983; KARPOV, PREOBRAZHENSKAYA and MIRZABEKOV 1984).

Recent, indirect studies have focused attention upon a possible relationship among histones, nucleosomal organization in chromatin and position effect variegation in Drosophila. Essentially, two sorts of experiments are pertinent: (1) Deletions of the histone gene region of *Drosophila melanogaster* (39DE) survive as heterozygotes and are associated with increased gene expression in variegated tissues due to position effect (MOORE, SINCLAIR and GRIGLIATTI 1983; MOORE *et al.* 1979). (2) Butyrate-feeding experiments, possibly associated with histone hyperactylation (SEALY and CHALKLEY 1978), similarly are associated with increased gene expression in variegated tissues due to position effect (MOTTUS, REEVES and GRIGLIATTI 1980).

In attempting to examine the effect of histone region deletion heterozygosity on the rosy locus position effect, we found that viability and infertility problems precluded test of the effect of *Df(2L)161*, a complete deletion of the histone gene region. However, with some difficulty, we were able to examine the effect of

TABLE 1

Percent phenocopy[a] as a function of HPP concentration and genotype

	$kar^2ry^{ps11136}/Tp(3)MKRS^b$		$kar^2ry^{+11}/Tp(3)MKRS^c$		$ry^+/Tp(3)MKRS^{b,c}$	
HPP (μg/ml)	*Df(2L)84*	*CyO*	*Df(2L)84*	*CyO*	*Df(2L)84*	*CyO*
0	0	0	0	0	0	0
30	10	25	0	0	0	0
50	35	76	0	0	0	0
70	76	96	0	0	0	0
90	97	100	0	12	0	38
110	100	100	0	16	5	67
130	100	100	0	65	20	91

[a] Percent phenocopy = $\frac{\text{Number of flies with rosy eye color}}{\text{Total number of flies}} \times 100$. Each percentage is based upon a sample size greater than 75 individuals.

[b] Progeny resulted from the cross *Df(2L)84/CyO;ry⁺/MKRS*♂♂ × +/+; $kar^2ry^{ps11136}/MKRS$ ♀♀.

[c] Progeny resulted from the cross *Df(2R)84/CyO;ry⁺/MKRS*♂♂ × +/+; $kar^2ry^{+11}/MKRS$ ♀♀.

Df(2L)84, which is believed to be a partial deletion of the histone gene region and which has a strong effect on increasing gene expression in variegated tissues (MOORE, SINCLAIR and GRIGLIATTI 1983). Starting with a leaky rosy mutant such as the position effect variant, $ry^{ps11136}$, response to HPP inhibition as measured by percent phenocopy is our most sensitive indicator of small changes in XDH levels (RUSHLOW and CHOVNICK 1984). Table 1 summarizes the results of appropriate crosses (as indicated) carried out on standard medium supplemented (as indicated) with various levels of HPP. A small but definite increase is seen in the amount of HPP required to produce a rosy mutant eye color in flies carrying the position effect mutant and heterozygous for *Df(2L)84* (column 2, Table 1) as compared to position effect mutant flies that do not carry the histone region deletion (column 3, Table 1). However, the ry^+-bearing flies emerging from the same cross in the same cultures are seen to exhibit a similar modification of rosy locus expression due to the presence of *Df(2L)84* (compare columns 6 and 7, Table 1). Since that ry^+ allele bears no relationship to the position effect mutation, $ry^{ps11136}$, another cross was carried out simultaneously in order to test the effect of *Df(2L)84* upon the HPP sensitivity of ry^{+11}-bearing flies. These data (columns 4 and 5, Table 1) provide additional evidence that the histone deletion is associated with increased rosy locus expression in the wild-type controls as well as in the position effect-bearing flies.

Another series of experiments were carried out to examine the effect of sodium butyrate on XDH levels of flies grown on standard medium and on standard medium supplemented with 0.2 and 0.4 M sodium butyrate. In these experiments we compared expression of the position effect mutants, ry^{ps1149}, $ry^{ps11136}$ and the wild-type alleles, ry^{+11}, ry^{+10} and ry^{+4}. Rosy locus expression was measured by CRM tests using rocket electrophoresis (RUSHLOW and CHOVNICK 1984). Because of excessively long developmental delays of all cultures on 0.4 M butyrate, this series was discontinued. While there was delayed growth on 0.2 M butyrate, the CRM tests were completed. In no case was there a difference in

CRM levels between flies grown on standard food and those grown on 0.2 M butyrate-supplemented food.

In view of our failure to find either a specific effect of *Df(2L)84* or of butyrate feeding on the expression of a position-affected rosy allele, we note additionally that we are able to confirm that both of these tests did produce an increase in white locus expression in the eye tissue of $In(1)w^{m4}$ flies. Although our observations on $In(1)w^{m4}$ confirm the earlier reports (MOTTUS, REEVES and GRIGLIATTI 1980; MOORE, SINCLAIR and GRIGLIATTI 1983), we are led to suggest that caution be exercised in interpretation of these observations. The experiments with variegated morphological phenotypes are essentially observations with no appropriate controls. In the case of the histone deletion study, we note the developmental delay and reduced viability and reproduction of flies carrying these deletions and the abnormal cultures that result. The underlying basis for the increased expression associated with both position effect and wild-type controls in our experiments cannot be easily deduced from these studies. Moreover, we note that butyrate also leads to developmental delay. Our failure to see a butyrate effect with rosy may relate either to our restriction of the study to a low butyrate concentration or to the possibility that the CRM test is insensitive. Finally, we note that our failure with these indirect experiments should not be considered as evidence against mechanisms of position effect involving chromatin organization. Rather, we believe that more direct, molecular studies are required.

DISCUSSION

Early classic studies (reviewed in LEWIS 1950; BAKER 1968; SPOFFORD 1976) demonstrated that the position-affected gene was itself not mutated. This inference was drawn from studies that separated the gene in question from the heterochromatic breakpoint either by a recombination event or by further rearrangement. In each case, such separation resulted in restoration of an apparent wild-type allele insofar as might be defined with a morphological phenotype. In the present case, no effort has been made to physically separate the position-affected rosy alleles from their respective heterochromatic breakpoints. However, we have taken advantage of the availability of overlapping clones of DNA that span the region surrounding the rosy locus (BENDER, SPIERER and HOGNESS 1983), as well as clones of DNA that represent internal sections of the rosy locus (B. COTE, W. BENDER and A. CHOVNICK, unpublished results), and the present report locates the position effect breaks on the DNA map to be outside the boundaries of the rosy locus DNA. Coupled with the observation that the XDH peptide product of a position-affected rosy allele is normal (RUSHLOW and CHOVNICK 1984) we are able to provide molecular confirmation of this classic notion of position effect.

Early notions concerning the mechanism of somatic variegation associated with heterochromatic position effect derive from the apparent "all or none" phenotypic effects inferred in several cases. Although most thinking (BAKER 1968; SPOFFORD 1976) on the subject has assumed a functional inactivation model, SCHULTZ (1936) proposed a somatic gene loss model of position effect that could not be eliminated by classical experimental approaches. Support for this model

comes from an interpretation of observations relating loss of rDNA compensatory response in rearrangement genotypes that exhibit position effect variegation (PROCUNIER and TARTOF 1978). However, HENIKOFF (1981), investigating a rearrangement exhibiting somatic variegation of the 87C heat shock puff, was able to present strong evidence against the somatic gene loss model. In the present case, in which we are dealing with a gene that functions principally in polytene tissue, very serious consideration had to be directed to the gene loss model. However, the present study, making use of molecular technology, eliminates the underreplication model. Moreover, evidence is presented that serves to focus future experimental directions to examination of pretranslational models of position effect.

This investigation was supported by research grant, GM-09886, (A. C.) and GM-28630 (W. B.) from the Public Health Service. C. A. R. was supported by predoctoral training grant, GM-07584, from the Public Health Service. The authors are grateful to ERICK FYRBERG of Johns Hopkins University for providing the 79B actin gene probe.

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Corresponding editor: T. C. KAUFMAN