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Is *Drosophila* dAT on the Y Chromosome?

(mutants/ultracentrifugation/embryos)

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ABSTRACT DNA isolated from *Drosophila melanogaster* embryos contains measurable amounts of dAT, the copolymer of deoxyadenylate and deoxythymidylate. In wild-type (XX, XY) embryos, dAT constitutes 4% of the total DNA. In embryos containing extra Y chromosomes, or even extra portions of Y chromosomes, dAT constitutes as much as 7% of the total. The enrichment for dAT is dependent upon the number of extra short arms of the Y chromosome (Y^S) or long arms (Y^L) of the Y chromosome per individual. This enrichment of dAT dependent on the Y chromosome may be interpreted in several ways. The most straightforward interpretation is that dAT is present in high concentrations on the Y chromosome.

dAT, the copolymer of deoxyadenylate and deoxythymidylate, was first discovered in DNA purified from testes of the crab, *Cancer borealis* (1). Since then it has also been found in *Drosophila melanogaster* embryos (2, 3) and in mitochondria from petite mutants of yeast (4). While dAT contains almost entirely adenine and thymine residues, their arrangement in the copolymer is apparently species-specific. For instance, crab dAT is almost exclusively the alternating copolymer, d(AT) (5), while *Drosophila* dAT is a mixture of both the alternating copolymer and the apposed copolymer, dA·dT (3). Because of its comparatively simple base-sequence, dAT represents an unusual hereditary signal. In spite of its simplicity, its function has not been determined.

Another as yet unsolved riddle is the matter of cytological localization. Do crab dAT and fly dAT occur in the nucleus, mitochondria, or elsewhere? The literature contains supporting evidence for each of these possibilities (2, 3, 6, 7). We study here the relationship between chromosome dosage and dAT concentration in *Drosophila melanogaster* embryos.

MATERIALS AND METHODS

Collection of Embryos. Embryos were produced and collected by modifying and extending the technique of Mitchell and Mitchell (8). Half-pint (200 ml) culture bottles containing cornmeal-agar medium were inoculated with adult flies and cultured at 26°C. Young flies began to emerge 9 days after the culture had been started, and reached optimal population density at 10–11 days of culture age. Adult flies from 60–65 bottles, usually 20–25 g, were emptied into a large plexiglass cage. One or two food dishes were placed in each cage. The food dishes were filled with cornmeal-agar medium, enriched with an additional 20 g of agar per liter. Dishes were stored at 4°C and covered with moist bakers' yeast (Fleischman's) before use. Cages were incubated at 26 ± 0.5°C. The food was changed at 2- to 16-hr intervals, and the eggs were collected. Eggs, yeast, and dead flies on the food dish were swept from the cornmeal surface, in a stream of cold water, onto a two-level screen. The upper screen was two layers of cheesecloth; the lower screen was a 100-mesh flour sieve. Flies and particles of cornmeal were retained on the lower screen, yeast was washed through both screens. The eggs were washed with cold tap water onto a Büchner funnel fitted with Whatman no. 1 filter paper, and air dried. The eggs were examined under a dissecting microscope for contamination, and weighed. This collection procedure had no effect on egg viability.

Genetic Methods. Compound XX and XY chromosomes, in which two homologous elements shared one centromere, and Y chromosome fragments, in which portions of the Y chromosome were lost, were studied. Strains containing attached X ($\hat{X}\hat{X}$), attached X, Y ($\hat{X}\hat{Y}$); attached X, long arm of Y ($\hat{X}\hat{Y}^L$); attached X, short arm of Y ($\hat{X}\hat{Y}^S$); long arm of Y (Y^L); short arm of Y (Y^S); and attached short arm of Y($Y^S \cdot Y^S$) chromosomes were used. The strains analyzed in these experiments are listed below. The unusual chromosomes are described by the conventions of Lindsley and Grell (9).

	♀♀	Description	♂♂	Description
1.	XX	Oregon R	XY	Oregon R
2.	$\hat{X}\hat{X}$/Y	ywf: =/Y	$\hat{X}\hat{Y}$/Y	$Y^S X \cdot Y^L$, In (1) EN,yvfcar/Y
3.	$\hat{X}\hat{X}/Y^S$	yf: $=/Y^S$	$\hat{X}\hat{Y}^L/Y^S$	$g^2B \cdot Y^L/Y^S$
4.	$\hat{X}\hat{Y}^S\hat{X}\hat{Y}^S$	yIn(1)Δ49vf·Y^S	$\hat{X}\hat{Y}^S/Y^L$	yIn(1)Δ49vf·Y^S/sc·Y^L
5.	$\hat{X}\hat{X}/Y^S \cdot Y^S$	yf: $=/Y^S \cdot Y^S$	$\hat{X}\hat{Y}^L/Y^S \cdot Y^S$	yvf·$Y^L/Y^S \cdot Y^S$
6.	$\hat{X}\hat{X}/Y^L$	C(1)RM y^2w^sbb/sc·Y^L	$\hat{X}\hat{Y}^S/Y^L$	yIn(1)Δ49 vf·Y^S/sc·Y^L

Stocks *1–5* were obtained from Dr. Burke Judd. Stock-*6* females were constructed with $\hat{X}\hat{X}/0$ females and $\hat{X}\hat{Y}^S/Y^L$ males.

P. $\hat{X}\hat{X}/0$ ♀♀ C(1) RM y^2w^sbb x $\hat{X}\hat{Y}^S/Y^L$ ♂♂
yIn(1)Δ49vf·Y^S/sc·Y^L

F_1. $\hat{X}\hat{X}/Y^L$ ♀♀ C(1) RM y^2w^9bb/sc·Y^L

Stock *6* consisted of F_1 females plus stock-*4* males.

In some strains, for instance, $\hat{X}\hat{X}Y$, $\hat{X}\hat{Y}Y$, both males and females carried extra Y chromosomes or extra Y-chromosome fragments. In other strains, for instance, $\hat{X}\hat{X}Y^S$, $\hat{X}\hat{Y}^LY^S$, only females carried extra Y-chromosome fragments.

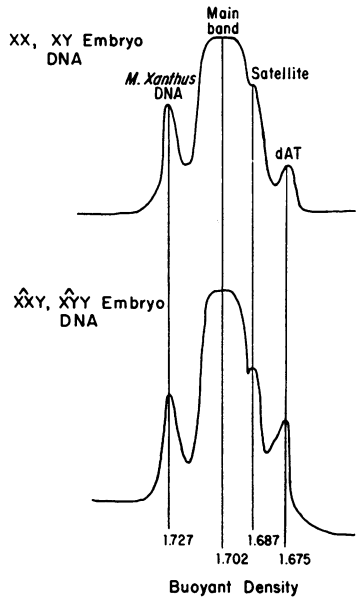

FIG. 1. Densitometric tracings from CsCl equilibrium centrifugation of *Drosophila* DNA; XX, XY and X̂XY, X̂YY DNA preparations were compared. 1.0 μg of *M. xanthus* DNA was used as a density marker. The main band = 40–42% G + C; the satellite = 28–30% G + C; dAT <5% G + C.

Purification of DNA. Fresh or frozen embryos (2–5 g) were dechorionated in 200 ml of half-strength Chlorox for 5 min, collected on a Büchner funnel, rinsed with 1 liter of distilled water and air-dried. All subsequent steps were at 0–4°C. The dried embryos were homogenized with 40 ml of 75 mM NaCl–30 mM EDTA, tetrasodium salt (pH 7.5) (NaCl–EDTA), with six strokes of a Teflon pestle driven by a 1/4-inch (0.6-cm) electric drill at full speed. The homogenate was filtered through four layers of cheesecloth into chilled centrifuge tubes. The homogenizer and gauze were rinsed with an additional 40 ml of NaCl–EDTA. The pooled filtrate was centrifuged at 2000 × *g* for 10 min and the supernatant was discarded. The pellet, consisting of nuclei, unbroken cells, and groups of cells, was suspended in 10.0 ml of fresh NaCl–EDTA, and homogenized with 10 strokes of a Dounce homogenizer, equipped with a tight-fitting, size-B pestle. The volume was adjusted to 40 ml with NaCl–EDTA, and the homogenate was centrifuged at 2000 × *g* for 10 min. The crude nuclear pellet was lysed in 0.01 M Tris·HCl (pH 8.0), and centrifuged at 2000 × *g* for 10 min. DNA was purified from the gelatinous pellet by a method adapted from that of Marmur (10). The pellet was suspended in 5 ml of 0.15 M NaCl–0.10 M Tris·HCl (pH 7.0). The solution was then made 2 M with respect to NaCl and 2% with respect to sodium dodecyl sulfate, and stirred for 5 min. An equal volume of chloroform: 2-octanol 24:1 was added. The mixture was shaken for 10 min and then centrifuged in a clinical centrifuge (about 3000 rpm). The aqueous phase was separated and DNA was precipitated from it by the addition of two volumes of 95% ethanol. The DNA was dissolved in 0.1 × SSC [SSC is 0.15 M NaCl–0.015 M sodium citrate (pH 7.0)]. The DNA solution was treated with ribonuclease and α-amylase (11). 20 × SSC was added to a final concentration of 1 × SSC. The DNA solution was shaken with chloroform–octanol, reprecipitated, and dissolved in 0.1 × SSC.

Thermal Denaturation of DNA; Estimation of Drosophila dAT. Hyperchromicity, the increased absorbance that accompanies the denaturation of DNA (12), was measured at 260 nm, as a function of increasing temperature, with an automatic Beckman–Gilford recording spectrophotometer. Samples of embryo DNA (50 μg) were adjusted to a concentration of 20 μg/ml (0.50 A_{260}/ml) in 2.5 ml of 0.1 × SSC and treated with pancreatic RNase (0.01 ml; 1 mg/ml in 0.1 × SSC) at 37°C for 20 min. The solutions were pipetted into 10-mm (light path) quartz cuvettes, which were stoppered, and optical measurements were recorded as the temperature was raised from 37 to 85°C at a rate of about 1°C/2 min. Three samples and a blank were analyzed in each experiment.

Hyperchromicity was plotted as a function of temperature for all DNA samples. Correction was not made for the dilution due to thermal expansion. The transition in the range of 48–52°C was due to *Drosophila* dAT. The percentage hyperchromicity that occurred over this range was equated with the fraction of DNA as dAT.

Analytical Ultracentrifugation. Drosophila DNA samples were analyzed by their buoyant densities in CsCl (11, 13). *Myxococcus xanthus* DNA (ρ = 1.727), a gift from Dr. Charles Laird, was used as a standard. Solid CsCl (optical grade; General Biochemicals, Chagrin Falls, Ohio) was added to 4–10 μg of DNA in 0.20 ml of 0.1 × SSC. The final CsCl concentration was adjusted to 1.71 g/cc. Samples were centrifuged to equilibrium at 42,040 rpm in a Beckman model E ultracentrifuge (17 hr; 20°C) and photographed with ultraviolet optics on Kodak Commercial sheet film. The photographic negatives were traced with a densitometer. The base compositions of the resolved DNA fractions were calculated from their observed buoyant densities in CsCl (13).

Preparative Ultracentrifugation; Identification of Drosophila dAT; Estimation of Drosophila dAT. Hg^{2+} preferentially binds to native, A + T-rich DNA. This selective binding can be used to purify crab dAT from total DNA by preparative ultracentrifugation in Cs_2SO_4–$HgCl_2$ gradients (14). In our experiments, the Cs_2SO_4–$HgCl_2$ method was used to fractionate *Drosophila* DNA, to identify the dAT component, and to estimate the dAT component as percent of total DNA.

Stock solutions of embryo DNA [about 1 mg/ml in 5 mM $Na_2B_4O_7$ (pH 9.2) (14)], purified Cs_2SO_4 (15) ($\rho \approx 2$ g/cc in 5 mM $Na_2B_4O_7$), 5 mM $Na_2B_4O_7$, and $HgCl_2$ (100 μg/ml) were combined in predetermined ratios. Each sample con-

TABLE 1. *The effect of extra Y chromosomes on dAT percentage*

Embryo	% dAT (±SD)
Wild type (XX, XY)	3.8 ± 0.6 (4)
Extra Y (X̂XY, X̂YY)	6.3 ± 0.6 (3)*

Each value represents the average dAT measurement for the indicated strain. The number of DNA samples analyzed for each strain is listed in parentheses. Samples within each group do not differ significantly from one another (F = 1.09, n_1 = 5, n_2 = 17). However, the X̂XY, X̂YY samples differ significantly from the XX, XY samples (F = 92.2, n_1 = 1, n_2 = 22).

* $P < 0.01$.

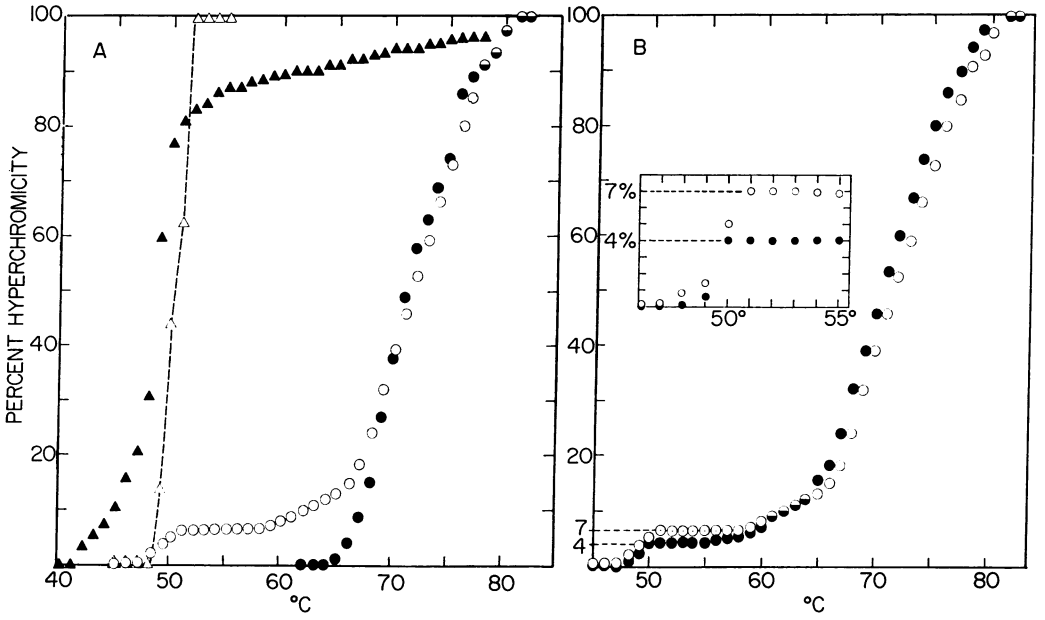

FIG. 2. Thermal denaturation of *Drosophila* and other DNAs in 0.1 × SSC. (*A*) Denaturation of purified *Drosophila* dAT (△), synthetic dAT purchased from Miles Laboratories (▲), $X\hat{X}Y$, $X\hat{Y}Y$ embryo DNA (○), and salmon-sperm DNA (●). *Drosophila* dAT denatures in the same temperature range as synthetic dAT. *Drosophila* DNA denatures stepwise, while salmon-sperm DNA denatures as one continuous function. (*B*) Denaturation of $X\hat{X}Y$, $X\hat{Y}Y$ (○) and XX, XY (●) DNA preparations. Both DNAs contain dAT. However, dAT reaches higher concentrations in $X\hat{X}Y$, $X\hat{Y}Y$ embryos. *Insert*, magnification of area around 50°C.

tained Cs_2SO_4 [ρ = 1.53 g/cc, as measured by refractive index (15)], 300 μg DNA, and 0.10–0.11 μmol of $HgCl_2$, in a final volume of 8.0 ml. Samples were pipetted into cellulose nitrate tubes for the Spinco 50 rotor, covered with 2 ml of water-washed mineral oil, and centrifuged (45,000 rpm; 20–24 hr) (16). 5-Drop fractions (about 0.15 ml) were collected from the bottom of each tube, diluted with 0.5 ml of borate buffer, and measured at 260 nm in a Cary model 14 spectrophotometer. The DNA fractions in each peak were pooled separately, dialyzed against two changes of 1 × SSC, then against two changes of 0.1 × SSC. Their identities were established by thermal denaturation and analytical ultracentrifugation. The A_{260} in the dAT peak (peak III, Fig. 3) was calculated as percent of total A_{260} for the DNA preparations.

RESULTS

Analytical ultracentrifugation

Densitometric tracings from CsCl equilibrium centrifugation of two different preparations of embryo DNA are illustrated in Fig. 1. One of the DNA samples was purified from wild-type (XX;XY) embryos. The other DNA sample was purified from "extra-Y" ($X\hat{X}Y$;$X\hat{Y}YY$) embryos. The tracings reveal that both embryo DNA preparations were resolved into three components in the ultracentrifuge: a major component (40–42% G + C), a less-dense "satellite" DNA (28–30% G + C), and dAT. This resolution is comparable to that reported (2, 3).

Thermal denaturation

The hyperchromicity of embryo DNA was 36%. The thermal denaturation profile of embryo DNA in 0.1 × SSC reveals that *Drosophila* DNA denatures stepwise (Fig. 2). One component denatures between 48 and 52°C, close to the observed denaturation range for synthetic dAT (Miles Laboratories). Since there is no further denaturation until 58°C, the 52–58°C plateau enables us to measure the percentage of hyperchromicity due to the denaturation of *Drosophila* dAT.

Fig. 2 also illustrates that dAT represents a higher percentage of the total hyperchromicity in DNA from extra-Y embryos than in DNA from wild-type embryos.

Experimental analyses of different wild-type and extra-Y DNA preparations are tabulated in Table 1. The differences between wild-type and extra-Y preparations were analyzed by the *F-test*, and found to be significant at the 99% confidence level. Separate preparations of wild-type DNA did not differ significantly in dAT content. Separate extra-Y DNA preparations did not differ significantly in dAT content.

Table 2 summarizes the results of experiments in which eggs carried extra Y^S or Y^L chromosome arms. The experiments show that embryos carrying one extra Y^L, or two or more extra Y^S chromosome arms, have significantly higher dAT values than do control embryos. When female embryos contain two Y^S, and male embryos contain one extra Y^S, the percentage of dAT is increased by 60–75% over control embryos. The measured values are significant at the 99% confidence level.

Preparative ultracentrifugation

Drosophila DNA was resolved into four fractions by centrifugation in Cs_2SO_4–$HgCl_2$ gradients (Fig. 3). The identity of each fraction was established by analytical ultracentrifugation in CsCl, and by thermal denaturation. Fraction I was identified as a previously unreported satellite DNA under the main band (ρ = 1.704), fraction II was identified as satellite DNA (ρ = 1.687), fraction III was identified as dAT (ρ = 1.675), and fraction IV was identified as the main component (ρ = 1.700).

Fraction I is an unusual DNA; its G + C content cannot be inferred from isopycnic banding data alone. AT-rich DNAs are usually dense in Cs_2SO_4–$HgCl_2$ gradients, but light

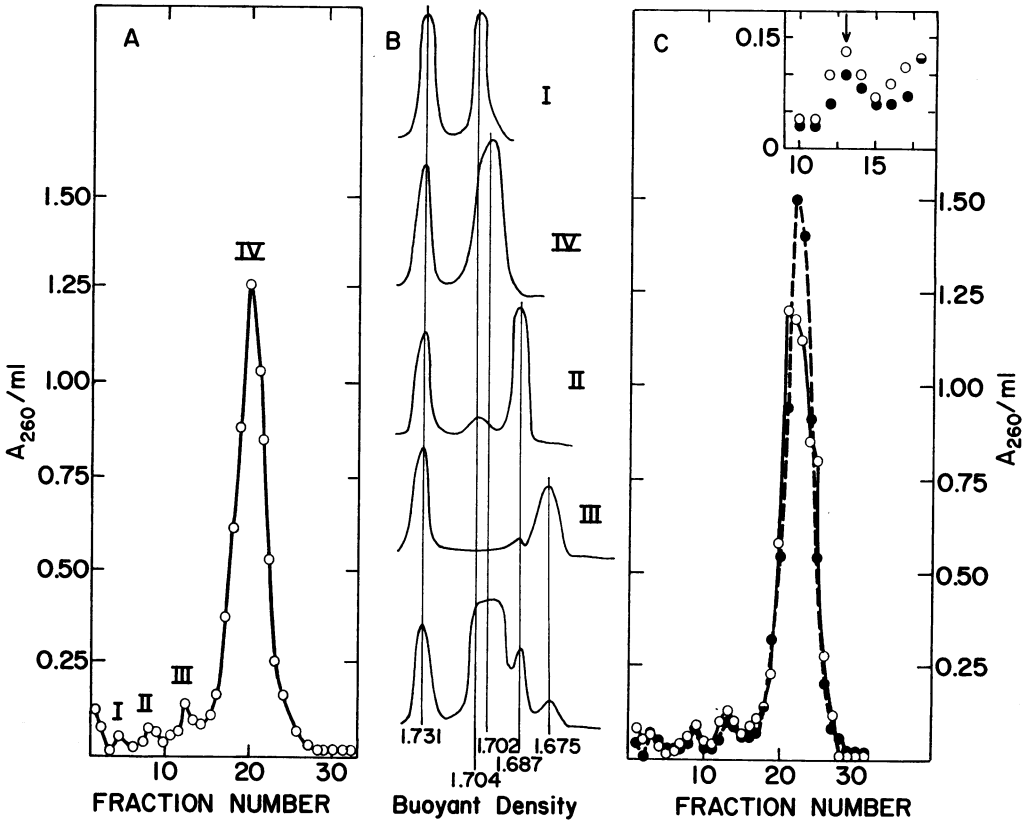

FIG. 3. Isopycnic banding of *Drosophila* DNA in preparative Cs_2SO_4–$HgCl_2$ gradients. (*A*) The banding pattern of $X\hat{X}Y$, $X\hat{Y}Y$ embryo DNA. Embryo DNA is resolved into four bands in these gradients. (*B*) The identity of the DNA bands. Peak fractions of each band were pooled separately, dialyzed, and identified by analytical ultracentrifugation in CsCl gradients. *Micrococcus luteus* DNA (ρ = 1.731) was used as a density standard. Fraction III was dAT. (*C*) Comparison of $X\hat{X}Y$, $X\hat{Y}Y$ (○) and XX, XY (●) DNA preparations in Cs_2SO_4–Hg^{2+} gradients. Background absorbance (A_{260} = 0.08) was subtracted from each fraction. Both DNAs contain dAT (fraction III). *Insert,* magnification of area around dAT peak.

in CsCl gradients (14, 17, 18). Since fraction I is dense both in Cs_2SO_4–$HgCl_2$ and in CsCl, it seems likely that its anomalous behavior can be attributed to some factor other than G + C content [for instance, an unusual base sequence or the presence of minor bases (15)].

TABLE 2. *Effect of extra Y^S or Y^L chromosome arms on dAT percentage*

Embryo	% dAT (±SD)
Wild type (XX, XY)	3.8 ± 0.6 (4)
1 extra Y^S $\left(\frac{X\hat{X}}{Y^S}, \frac{X\hat{Y}^L}{Y^S}\right)$	3.9 ± 0.4 (1)
2 extra Y^S $\left(\frac{X\hat{Y}^S}{XY^S}, \frac{X\hat{Y}^S}{Y^L}\right)$	5.4 ± 0.6 (3)*,[a]
3 extra Y^S $\left(\frac{X\hat{X}}{Y^S.Y^S}, \frac{X\hat{Y}^L}{Y^S.Y^S}\right)$	6.5 ± 0.6 (2)*,[b]
1 extra Y^L $\left(\frac{X\hat{X}}{Y^L}, \frac{X\hat{Y}^S}{Y^L}\right)$	6.3 ± 0.4 (2)*,[c]

Each value represents the average dAT measurement for the indicated strain. The number of DNA samples analyzed for each strain is listed in parentheses. Samples within each group do not differ significantly from one another. [a] F = 2.40, n_1 = 18; [b] F = 1.29, n_1 = 4, n_2 = 17; [c] F = 0.79, n_1 = 4, n_2 = 14. However, $\frac{X\hat{Y}S}{X\hat{Y}S}$, $\frac{X\hat{Y}^S}{YL}$; $\frac{X\hat{X}}{Y^S.Y^S}$, $\frac{X\hat{Y}^L}{Y^S.Y^S}$; and $\frac{X\hat{X}}{Y^L}$, $\frac{X\hat{Y}^S}{Y^L}$ samples differ significantly from the XX, XY samples. [a] F = 38.2, n_1 = 1, n_2 = 23; [b] F = 73.7, n_1 = 1, n_2 = 21; [c] F = 81.5, n_1 = 1, n_2 = 18. * $P \ll 0.01$.

Fraction III, *Drosophila dAT*, is 6% of the total A_{260} in DNA from extra-Y embryos, but only 3.5% of the total A_{260} in DNA from wild-type embryos (Fig. 3). Thus, the observed correlation between the Y chromosome and percentage of dAT, based upon thermal denaturation experiments, was confirmed by preparative ultracentrifugation.

DISCUSSION

These experiments demonstrate a correlation between the presence of the Y chromosome and the concentration of dAT in *Drosophila* embryos. However, the Y chromosome is not the only element involved, because XO adult males, genetically deprived of their Y chromosomes, also contain measurable amounts (about 2%) of dAT (unpublished results).

From the observed correlation between Y-chromosome dosage and dAT concentration, it is tempting to conclude that dAT is present in high concentration on the Y chromosome. However, the correlation is also compatible with alternative interpretations. Among these are the following: (*a*) The Y chromosome carries a factor that stimulates dAT synthesis, or retards dAT degradation. (*b*) The Y chromosome is associated with the maternal packaging and transmission of extranuclear dAT. "Extra-Y" female flies in these experiments carry an entire Y chromosome or at least one Y^S or Y^L chromosome. Our results could be explained by the hy-

pothesis that mothers carrying the Y chromosome, or part of the Y chromosome, package and transmit excessive amounts of dAT to their embryos. In our opinion, the most reasonable interpretation of our findings is that the Y chromosome carries high concentrations of dAT.

NOTE ADDED IN PROOF

Recent experiments indicate that crab dAT is nuclear because it is present in nuclei, but not in mitochondria (19,20).

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