HISTONE CONTENT OF CHROMOSOMAL LOCI ACTIVE
AND INACTIVE IN RNA SYNTHESIS

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INTRODUCTION

It has been reported that histones inhibit DNA-dependent RNA synthesis in vitro (5, 15) and these results have been taken as support of the hypothesis (34) that histones function as regulators of gene activity in vivo. One necessary corre-late of this hypothesis is that different cell types contain different amounts and/or different kinds of histones or that the histone-DNA interactions differ in different tissues. However, biochemical analyses have not conclusively demonstrated tissue specificity of histones (18). This is perhaps due to

their heterogeneity, to their tendencies to aggregate and complex with other proteins, and to the lack of a precise definition which sets histones apart from other classes of proteins (for reviews concerning the properties of histones see references 16 and 17). Another complicating factor in such analyses is that, with the exception of nucleated erythrocytes in which a unique histone has been reported (28), all of the cell types so far examined have been engaged in RNA synthesis, and the differences between them, if any, may be slight. Therefore, it would seem desirable to look for histone variation in systems in which the differences in genetic activity are maximized, preferably situations in which DNA-dependent RNA synthesis is occurring at a high rate compared with situations in which it is not occurring at all. Such an "on-off" system is found in the polytene chromosomes of the salivary gland nuclei of *Drosophila* and other flies. RNA synthesis in these chromosomes is most intense at specific regions called puffs because of their expanded nature (8, 36, 37). Puff patterns are tissue specific (7) and puff formation varies in a predictable fashion through development (8). Therefore, puff regions seem to have the properties of active genes. DNA bands are thought to be inactive genes, and contain no cytochemically detectable RNA. Therefore, if histones do in fact control gene action, we might expect to find differences in amount or kinds of histones between puffs and DNA bands. Moreover, since different DNA bands probably represent different genes, we might also expect to find different amounts or types of histones associated with different bands, as has been reported in the salivary gland chromosomes of *Sciara* (11). For these reasons we have undertaken quantitative cytochemical analysis of the end of chromosome 2 of *D. virilis* by microdensitometry of Feulgen- and alkaline fast green-stained salivary gland squashes. This region includes an RNA-containing puff, which actively incorporates radioactive RNA precursors, and three bands which do not contain any cytochemically detectable RNA and which incorporate little or no radioactive ribonucleotides (36, 37). We have also measured a complex terminal region of the chromosome which includes the telomere, a region of indistinct banding (heterochromatin?) and occasionally a small subterminal puff.

MATERIALS AND METHODS

Salivary glands were dissected from third instar larvae and squashed in 45% acetic acid. Coverslips were removed after freezing on dry ice and the squashes were postfixed for 1–3 hr in 10% formalin at 4°C. One set of slides was hydrolyzed in 1 N trichloroacetic acid at 60°C for 14 min and stained by the Feulgen procedure for DNA. Unhydrolyzed controls treated in water at 60°C for 14 min showed no detectable staining, and slides extracted with DNase were also negative when stained by this method. Another set of slides was extracted in 5% trichloroacetic acid at 90°C for 15 min to remove nucleic acids, rinsed in 70% ethanol followed by water adjusted to pH 8.1, and stained in 0.1% fast green at pH 8.1 for 1 hr (3). The slides were then rinsed briefly in three changes of water at pH 8.1 and dehydrated in the usual manner. Controls for the alkaline fast green reaction consisted of slides which were treated in 0.2 N HCl for 4 hr at room temperature after squashing but *before* formalin fixation to extract histones and of slides treated in 90° H_2O for 15 min as a control for nucleic acid removal. Both controls showed no detectable staining. Nitrous acid deamination (5% $NaNO_2$ + 5% trichloroacetic acid, 15 min, twice) (38) performed either before or after nucleic acid removal also completely abolished alkaline fast green staining.

The slides were mounted in refractive index oils and the amount of dye bound was determined by the method of photographic plate microdensitometry (19, 31). Large, well-spread regions of chromosome 2 were photographed with essentially monochromatic light (λ = 570 mμ for Feulgen, 590 mμ for alkaline fast green) on Kodak M-plates. Magnification at the plate was 2500. The photographs were developed in Kodak D-19 developer for 5 min. The negatives were scanned with a Joyce Loebl 111-B recording microdensitometer.[1] Deviations from linearity in the photographic process and variations between plates were determined by including on each negative the image of a rotating disc cut to give extinctions from 0.10 to 1.0 in steps of 0.10. Between 20 and 30 longitudinal tracings were taken at 0.2 μ intervals across each chromosome (Fig. 2), and a tracing of the background adjacent to the chromosome

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[1] Joyce Loebl and Company, Ltd. Purchased from National Instrument Laboratories, Inc. Rockville, Maryland.

TABLE I

DNA (Feulgen) and Histone (Trichloroacetic Acid Alkaline Fast Green) Contents of Five Regions of Chromosome 2 of Drosophila Virilis

Values are means ± standard errors in arbitrary photometric units.

Region	N	Feulgen DNA	Fast green histone	Histone / DNA
Terminal	7	5797 ± 120	2034 ± 99	0.35
Band 1	7	1573 ± 38	600 ± 32	0.38
Band 2	7	1228 ± 24	457 ± 39	0.37
Band 3	7	1674 ± 62	564 ± 58	0.34
Puff	7	2530 ± 53	872 ± 68	0.34
Total	7	12798 ± 166	4527 ± 254	0.35

A partially hierachical two-way analysis of variance shows no significant differences $(0.5 < P < 0.75)$ between the ratios.
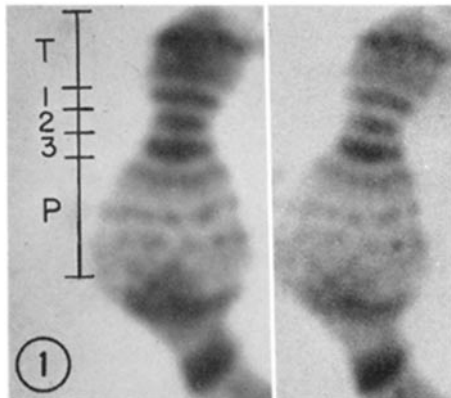

Figure 1 An end of chromosome 2 stained first with the Feulgen reaction for DNA and then destained and subsequently restained with the alkaline fast green reaction for histones. T, Terminal region; 1-3, Bands; P, Puff.

was included in every trace. The patterns of the tracings, especially when compared with the original plates, usually allowed unambiguous demarkation of the measured areas. Interbands and the rare ambiguous areas were arbitrarily included in the region above them except for the border between band 3 and the puff where expansion of the puff and lower extinctions made it necessary to include that interband in the puff (Fig. 2). The total amount of absorbing material was considered to be the sum of the weights of the cut-outs of each tracing after the above corrections had been made (33). The ratios presented in Table I (~ 0.35) accurately reflect the relative intensities of the two stains at the wavelengths chosen for measurement.
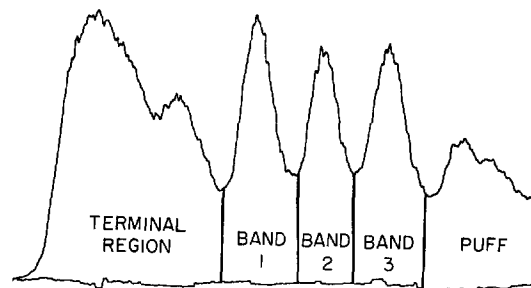

Figure 2 A sample densitometer tracing through a Feulgen-stained end of chromosome 2. The terminal region, which includes the telomere and occasionally a subterminal puff, shows some evidence of banding in this tracing.

RESULTS

Fig. 1 shows the regions measured at the end of chromosome 2 stained for DNA and for histone. The band patterns are identical in the two preparations (25, 32) and histone staining is easily detectable in the puffed regions. Fig. 2 shows sample densitometer tracings lengthwise through a Feulgen-stained chromosome. The measured regions can be clearly resolved. Although the chromosomes often were well stretched with wide spaces between some of the bands, the densitometer tracings through the chromosomes do not return to the base line (zero absorption), suggesting that both DNA and histone are found in interbands as well as in bands, and that both are continuous through the length of the chromosome at the level of resolution of the light microscope. In Table I, the means for Feulgen and fast green amounts (in arbitrary units) for the five regions

are presented, along with the fast green to Feulgen ratios. There are no significant differences between the ratios for any of the five regions.

DISCUSSION

The accuracy and limitations of the method of photographic plate densitometry have been discussed elsewhere (19, 31, 39). It is among the most accurate methods for microspectrophotometry of small biological objects. The quantitative validities of the Feulgen reaction for DNA (35) and of alkaline fast green for basic proteins (3, 12) have also been demonstrated.

Histones have been defined as basic proteins that at some time are associated with DNA (27). Cytochemically, histones in polytene chromosomes can be operationally defined as acid-soluble (0.2 N HCl) proteins which stain with alkaline fast green (and are, therefore, basic) only after removal of DNA (and are, therefore, associated with DNA). The cytochemical fraction studied by these techniques thus conforms closely to the generally accepted definition of histones. Whether all histones are stained by this method, especially after squashing in 45% acetic acid, is not known. Swift (37) found that prolonged extraction in 45% acetic acid had no effect on alkaline fast green staining in *Drosophila* polytene chromosomes. Studies on isolated nuclei have shown no marked reduction in alkaline fast green staining after extraction in 45% acetic acid for 1 hr under conditions where staining is abolished by 0.2 N HCl after as little as 15 min extraction (M. Gorovsky, unpublished observations). Moreover, proteins with a net positive charge (determined by acrylamide gel electrophoresis) were not extractable with 45% acetic acid from isolated *Vicia* nuclei under conditions where dilute inorganic acids extracted large quantities of basic proteins (21). Holtzman (24) found correlations between standard biochemical extraction procedures for various histone fractions and alkaline fast green stainability, and it has been known for some time that the predominantly lysine-rich somatic histones and the arginine-rich histones which replace them during spermiogenesis in some animals both stain with alkaline fast green (2, 14). Therefore, we feel that most if not all fractions of histones were stained by the methods employed here. However, extraction of histones by 30% acetic acid has been reported (1), and whether *any* histones are extracted or unstained by these techniques in

Drosophila salivary gland chromosomes can only be determined by correlated biochemical and cytochemical fractionations not currently practical with this material.

Many mechanisms have been proposed to explain the altered state of histone or changes in DNA-histone interaction which may occur during the transition of DNA from the inactive condition to an active participation in RNA synthesis. Bonner and Huang (15) have suggested that only DNA which is not complexed with histones is responsible for RNA synthesis in pea embryo chromatin. Clearly, this is not the case in *Drosophila* since the same amount of histone staining per unit DNA-Feulgen was found in the puff as in the three-banded regions. Frenster (22) has suggested that nuclear polyanions such as RNA or phosphoproteins may interact with histones and alter their association with DNA, and thus may allow RNA synthesis to proceed. Both RNA and nonhistone protein are present in large quantities in puffs (36). Although RNA is removed by the trichloroacetic acid extraction used here, similar ratios of histone to DNA amounts for the five measured regions are also found if DNase is used instead of trichloroacetic acid (23). Studies on the mechanisms of dye binding by ionic linkages (30, 33, 35) strongly suggest that anionic molecules would compete with the fast green for binding sites and thereby decrease staining intensity. Thus, although they are present in large quantities in puffs, it is unlikely that RNA or acidic proteins are intimately associated with histones. However, the 45% acetic acid treatment or subsequent preparative procedures may alter the naturally occurring physical associations between histones and other macromolecules occurring in the puff, releasing reactive groups which are bound in vivo to complex with the dye after squashing.

On the basis of studies with reconstituted nucleohistone, Bonner and his co-workers (26) have also suggested that certain histone fractions, when associated with DNA, allow RNA synthesis to occur, whereas others do not. Therefore, puffs might differ from bands in histone types rather than amounts. Since different fractions of histones have different isoelectric points and different charge densities, they would be expected to bind different amounts of fast green (30). The similarities in the ratios for the puff and the three bands argue against the existence of any major differences in histone types between these regions. In

addition, nitrous acid deamination, which removes the amino groups of lysine but not the guanidino groups of arginine (20), reduces the staining in all regions below detectable levels; this suggests that no marked differences occur in lysine:arginine ratios in these regions. Our methods would not detect small differences in histone composition or alterations in the physical association of DNA and histones unless these markedly affected the staining through one of the mechanisms suggested above. Similarly, if RNA synthesis is controlled by a slight variation in the total level of acetylation of histones as has been recently suggested (6), these differences would not be reflected in alkaline fast green: Feulgen ratios.

The results obtained here may be criticized because we have only measured the histone:DNA ratios in the puff region while it was in the active or puffed condition. This ratio may be different from that obtained when this region is in the banded state. Two observations suggest that this is not the case: (a) the ratio obtained for the puff is identical with that obtained for the three-banded loci, making it unlikely that any euchromatic band will show a unique ratio; (b) Swift (37) measured the ratio of histone staining in band 3 and the puff region, when the puff region was in the banded condition, and found that the ratio was the same as it was when the region was puffed; this observation indicates that there is no change in histone amount with the onset of puffing.

Variations in fast green:Feulgen ratios have been reported (a) between nuclei of the same cell type in different functional states (10), (b) between cells of the same tissue in different states of growth (13), (c) in two different nuclei within a protozoan cell (4), and (d) between two haploid genomes within the same nucleus (9). In the first two cases, large differences in acidic, nonhistone proteins may have decreased the alkaline fast green staining, while the second two cases both involved entire nonfunctioning (heterochromatic) genomes which, unlike the euchromatic loci studied here, may have different histone compositions.

In conclusion, we feel that there are no marked quantitative or qualitative differences in alkaline fast green staining associated either with different states of RNA synthetic activity or with different loci of *Drosophila* polytene chromosomes. For the technical reasons discussed above, these findings must be interpreted cautiously. However, they do suggest that, even in extremely different states of RNA synthetic activity, histone differences, if present at all, may be quite subtle.

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