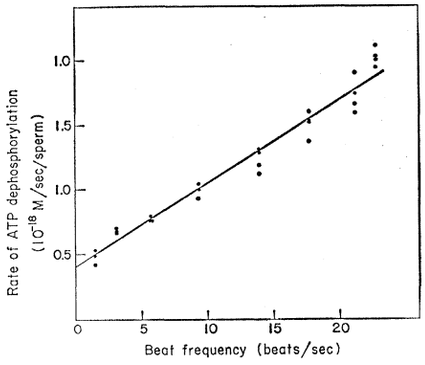

Fig. 2. Dephosphorylation of ATP by sperm suspensions at various beat frequencies obtained by varying the ATP concentration. Each point represents a single measurement of the rate of ATP dephosphorylation. The line was obtained by the method of least squares, and has a slope of 0.065×10^{-18} mole per beat per sperm.

ATP under these conditions does not vary with ATP concentration in the manner expected for a single enzyme obeying Michaelis-Menten kinetics. Most likely, these sperm preparations contain several active adenosine triphosphatases, and other methods will be needed to individually characterize them. In Fig. 2, the results from experiments with four sperm preparations are presented by plotting the rate of ATP dephosphorylation against the average beat frequency observed at the same ATP concentration. This mode of presentation suggests that a portion of the adenosine triphosphatase activity may be independent of the beat frequency, but that the major portion may be proportional to the beat frequency, with approximately 0.65×10^{-19} mole of ATP dephosphorylated per beat by each spermatozoon.

The results in Fig. 2 are too widely scattered to provide any firm evidence for a tight coupling between movement and ATP dephosphorylation. Such coupling seems unlikely in these preparations, since only a fraction of the spermatozoa in the reaction mixture are swimming actively, and variations in this fraction do not appear to be correlated with the rate of dephosphorylation. Evidence is also lacking that it is actually the dynein component that is responsible for the ATP dephosphorylation which is proportional to the beat frequency. These problems require further study. However, if the sole source of energy for flagellar movement is the indicated dephosphorylation of 0.65×10^{-19} mole of ATP per beat, a sea urchin spermatozoon which normally beats at 30 beats per second at 16°C will use approximately 2×10^{-18} mole of ATP per second. Since the work that it performs against the external viscous resistance appears to be about 3×10^{-7} erg per second (*5*), it must convert the free energy of ATP to external work at a rate equivalent to nearly 4 kcal per mole of ATP. This compares favorably with a value of 5.9 kcal per mole of creatine phosphate estimated for the work output of muscle (*12*).

The dynein molecules obtained from *Tetrahymena* cilia form chains with a spacing in vitro of 140 Å (*1*), while estimates of their spacing in vivo of 160 to 200 Å have been obtained (*13*). If sea urchin spermatozoa have a dynein with a configuration and localization similar to that of *Tetrahymena* cilia, a chain of dynein molecules with 140- to 200-Å spacing extending the length of a sperm flagellum, 42 μ (*5*), will contain 2 to 3×10^3 dynein molecules; and 18 chains, corresponding to one for each arm of each outer doublet fiber in the ciliary cross-section (*8*), will contain 3.6 to 5.4 $\times 10^4$ molecules, or 0.6 to 0.9×10^{-19} mole of dynein. Although a more direct and precise estimate of the number of dynein molecules in a sea urchine spermatozoon would be desirable, this estimate is close enough to the value of 0.65×10^{-19} mole per beat obtained for the rate of ATP dephosphorylation to support the suggestion that each dynein molecule uses one ATP molecule during each beat cycle.

C. J. Brokaw

Division of Biology, California Institute of Technology, Pasadena

References and Notes

1. I. R. Gibbons and A. J. Rowe, *Science* **149**, 424 (1965).
2. I. R. Gibbons, *J. Biol. Chem.* **241**, 5590 (1966).
3. M. A. Sleigh, *The Biology of Cilia and Flagella* (Pergamon, New York, 1963).
4. C. J. Brokaw, *J. Exp. Biol.* **40**, 149 (1963).
5. ———, *ibid.* **43**, 155 (1965).
6. ——— and L. Wright, *Science* **142**, 1169 (1963).
7. N. R. Silvester and M. E. J. Holwill, *Nature* **205**, 665 (1965).
8. I. R. Gibbons, *Arch. Biol.* **76**, 317 (1965).
9. H. Hoffman-Berling, *Biochim. Biophys. Acta* **16**, 146 (1955); S. Kinoshita, *J. Fac. Sci. Univ. Tokyo IV Zool.* **8**, 219 (1958); C. J. Brokaw, in *Spermatozoan Motility*, D. W. Bishop, Ed. (AAAS, Washington, D.C., 1962), pp. 269–278.
10. C. J. Brokaw, *Exp. Cell Res.* **22**, 151 (1961).
11. ———, *J. Exp. Biol.* **45**, 113 (1966).
12. F. D. Carlson, D. J. Hardy, D. R. Wilkie, *J. Gen. Physiol.* **46**, 851 (1963).
13. A. V. Grimstone and A. Klug, *J. Cell Sci.* **1**, 351 (1966).
14. Supported in part by PHS grants GM-6965 and GM-14613. I thank B. Benedict for assistance with this work, and Dr. G. Stephens for use of facilities at the University of California, Irvine.

3 February 1967

Histones in the Wild-Type and the Anucleolate Mutant of Xenopus laevis

Abstract. *A comparison of anucleolate mutant and wild-type tadpoles of* Xenopus laevis *reveals striking differences in histones. In the mutant, synthesis of histone fractions I and IIb is virtually absent and IIa is reduced, while fractions III and IV are significantly increased.*

Several studies have shown histones to be inhibitors in vitro of DNA-directed RNA synthesis (*1*). Moreover differential extraction of histone from its native association with DNA has resulted in the increased priming ability of the DNA (*2*). If we are to determine the role of histones in gene control, it would be desirable to relate the findings of these in vitro studies to known developmental alterations of gene readout, and to known repressed states of the nucleus. Condensed, or hetero, chromatin represents such a repressed state. Brown and his co-workers have shown that developmentally condensed chromatin in mealy bugs is genetically inactive (*3*) by virtue of repressed RNA synthesis (*4*). We have described histone differences in condensed and diffuse chromatin for this organism (*5*). The work of other laboratories also suggests a relationship between structural alteration of chromatin, genetic inactivity, and histone differences (*6*).

In the present study we have attempted to look at qualitative differences in histones associated with another nuclear alteration, the absence of nucleoli and nucleolar organizers in the anucleolate mutant of the African clawed toad, *Xenopus laevis*. This mutant (the homozygous recessive) lacks nucleoli, and is devoid of ribosomal RNA synthesis (*7*), but it manages to live to the swimming tadpole stage (*8*).

Sexually mature toads, phenotypically normal but heterozygous for the anucleolate condition (having one nucleous, rather than two), were injected with gonadotropin and mated. The resulting eggs were allowed to develop in dechlorinated tap water until they reached Nieuwkoop-Faber stage 28.

Approximately 1000 tadpoles were placed in 40 to 60 ml of water with 1 mc (10 to 36 mc/mmole) of sodium C^{14}-carbonate, 0.5 mg of penicillin per milliliter, and phenol red as *p*H indicator (*p*H 8). The tadpoles were

incubated for 3 days with daily exchange of the medium. Presumably this is an adequate interval to permit labeling of the histone fractions. By this time stages 40 to 42 were reached and one could discern characteristic morphological differences between the normal (homozygote and heterozygote) and mutant tadpoles. The tadpoles (stages 40 to 42) were then washed in tap water, and phenotypically normal tadpoles were separated from mutants. Nuclei were prepared from the labeled tadpoles. The tissues were homogenized with a Teflon pestle with 20 volumes of 0.0125*M* citric acid. Homogenates were then passed through three layers of cheese cloth and centrifuged for 5 minutes at 2000 rev/min in a swinging bucket rotor. The supernatant was removed and the pellet was washed twice with 0.125*M* citric acid/0.01 percent Tween 80, the sample being centrifuged under the same condition after each wash. The pellet was then washed with 0.1*M* tris (*p*H 7.3) to extract acidic protein, and the nuclei were examined under the phase contrast microscope. This procedure usually resulted in unbroken nuclei contaminated by some yolk granules. All preceding and subsequent steps were carried out at 0°C.

Histone was prepared from the tadpole nuclei by extraction with sulfuric acid. [For rationale see Murray (*2*).] The purified nuclei were syringed until homogenous in a small volume of 0.0125*M* citric acid and were then extracted with 10 percent sulfuric acid added in drops until *p*H 1.75 was reached. Extraction continued at *p*H 1.75 for 30 minutes with constant monitoring. The mixture was then centrifuged at 10,000 rev/min for 5 minutes. The supernatant was set aside and the residue was again extracted with sulfuric acid, this time to *p*H 0.6, and held for 30 minutes. The mixture was centrifuged at 10,000 rev/min for 5 minutes. The supernatants from both extractions were combined with 5 to 10 mg of calf thymus histone (prepared in our laboratory, or type II obtained from Sigma, London) and coprecipitated in 2.5 times their volume of ethanol at −20°C for from 1 to 3 days.

The resulting histone precipitate was collected and washed with ethanol three times, dried, and then dissolved in 20 ml of 8 percent guanidine hydrochloride.

Cation-exchange column chromatography was performed according to Satake *et al.* (*9*), with Amberlite CG-50 (100 to 200 mesh) as the resin and guanidine hydrochloride (GuHCl) as the eluent. The column size was approximately 30 by 60 mm. The dissolved histone was loaded on and eluted from the column at 12 to 14 ml/hour at room temperature. The charged column was washed overnight at this flow rate with 8 percent GuHCl until background levels of radioactivity were obtained. A GuHCl gradient of between 8 and 13 percent was created in a two-vessel system containing first 50

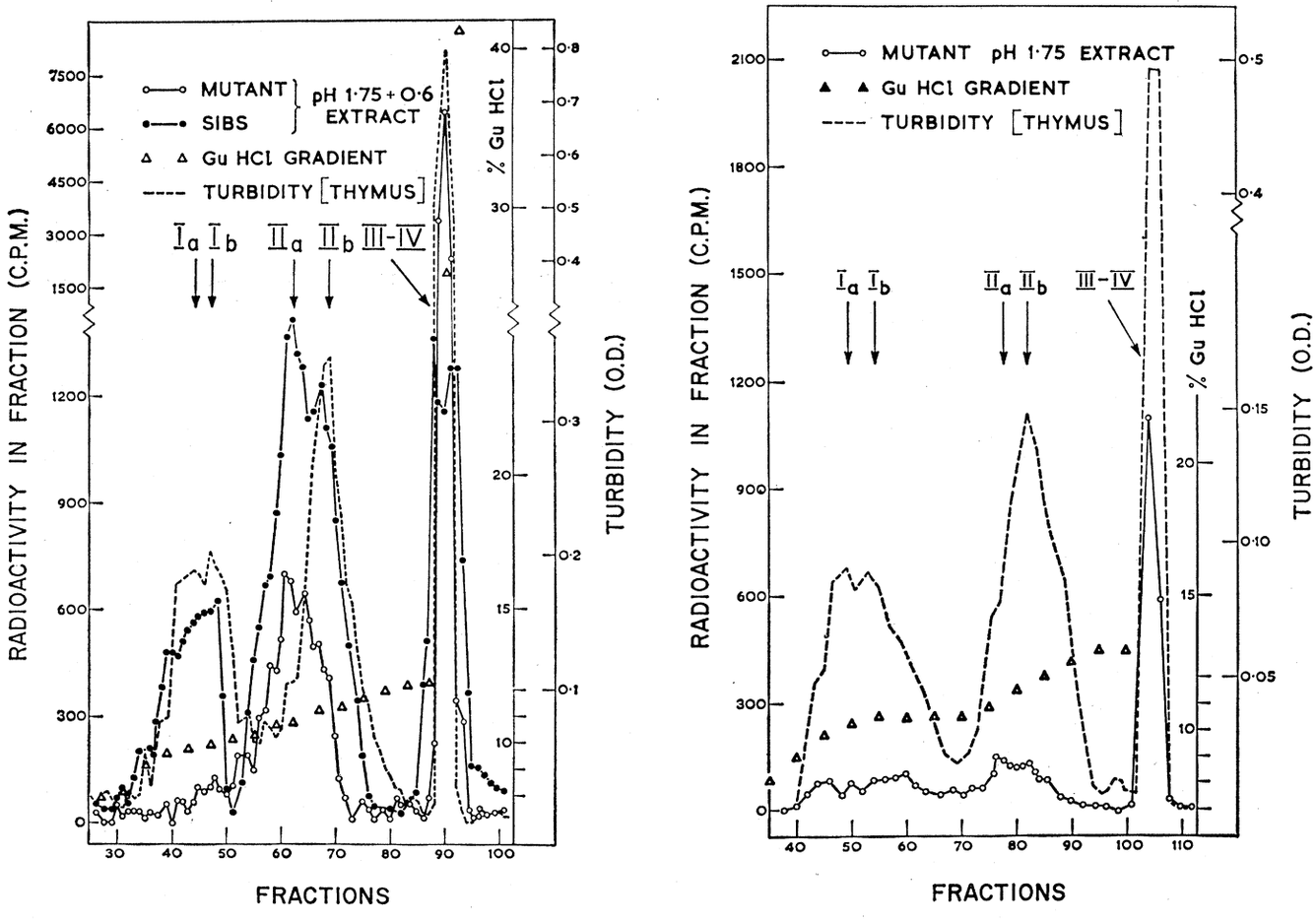

Fig. 1 (left). Comparison of C^{14}-labeled histones from anucleolate mutants of *Xenopus laevis* and their phenotypically normal sibs. In this experiment 357 heterzygous and homozygous wild-type (in a ratio of 2:1) and 164 homozygous mutant tadpoles were used. The anucleolate form lack newly synthesized fractions Ia, Ib, and IIb. Fig. 2 (right). Analysis of the *p*H 1.75 extractable histones of the anucleolate mutant. Labeled histone fractions were contributed by 284 C^{14}-labeled mutants, and analyzed as described in the text.

ml of 9 percent and 200 ml of 10.5 percent, and subsequently 100 ml of 10.5 percent and 300 ml of 13 percent GuHCl. After complete passage of the gradient the column was further eluted with 250 ml of 40 percent GuHCl.

The carrier thymus histones yielded the typical fraction peaks of that histone. A small runoff peak preceded the histone fractions designated as Ia, Ib, IIa, IIb, and III and IV [according to Murray's terminology (*2*)]. The discrimination of these fractions in the same pattern generally described in the literature (*2, 9*) (see Figs. 1 and 2) validates our histone extraction method.

Nonradioactive histone determinations were made turbidometrically by optical density at 400 mμ (in some instances at 300 mμ) when 1 ml of the effluent sample was combined with 3 ml of trichloroacetic acid (277 g/liter). The optical density was read after 30 minutes.

Radioactive histone determinations were made by adding a 1-ml fraction of the effluent sample to 10 ml of counting medium [after Chalkley and Maurer (*10*)]. In this way direct comparisons could be made between histones from two sources, since the radioactive histones did not measurably add to the turbidity measurement.

Figure 1 illustrates the results of series of experiments in which C^{14}-labeled histone from normal and mutant tadpoles was directly compared with calf-thymus histone. It should be noted that specific DNA activity in stage-42 mutants is 80 percent of that of their wild-type sibs. The curve for mutant histone has been adjusted to reflect the total DNA synthetic activity during the time of incubation, so that comparing the size of the peaks gives a more reasonable indication of quantitative difference in histone. Wild-type tadpoles follow the calf-thymus pattern rather closely with the exception of fractions IIa and IIb. Fraction IIa predominates in *Xenopus* and fraction IIb in calf thymus. The mutant tadpoles show an even more pronounced change. Fraction I appears to be completely missing. Fraction IIa appears reduced, and fraction IIb is either entirely missing or present in only small amounts. On the other hand, the peaks that appear to be fractions III and IV are significantly increased over both wild-type *Xenopus* and calf thymus.

The virtual absence of labeled fractions I and IIb from the anucleolate tadpoles is confirmed in experiments in which a *p*H 1.75 differential extract (*2*) was analyzed separately (Fig. 2). Under these conditions fraction IIa remains unextracted and cannot contribute to the chromatogram. In control experiments with identical fractions of the phenotypically normal sibs (not shown), good coincidence is found in the I and IIb range between turbidity (calf thymus) and radioactivity (*Xenopus*).

A separate set of experiments tends to indicate that cytoplasmic basic proteins (for example, those derived from ribosomes) cannot contribute to our results. When either mutant or wild-type *Xenopus* cytoplasmic fractions are prepared, extracted, and coprecipitated in a manner identical to the nuclear fractions above, no radioactivity is associated with the genuine thymus histone peaks. There are, however, high levels of radioactivity associated with the runoff peak, and with the gradient prior to the elution of fractions Ia and Ib.

Because of the good correspondence of the wild-type *Xenopus* fractions with the calf-thymus fractions, it is reasonable to infer that the lysine content of the calf-thymus fractions is a valid measure of the lysine content of the *Xenopus* fractions. It is likely, therefore, that the missing fractions (I and IIa) of the mutant tadpoles correspond to high and medium lysine histones, respectively.

The missing histone of fractions I and II in the anucleolate mutant tadpoles may represent either (i) stable or structural histone associated with the deleted heterochromatic nucleolar organizer, or (ii) chromosomal histone not being synthesized in the absence of the nucleolus. Wallace and Birnstiel (*8*) report that the chromatin of the nucleolar organizer regions consists of only a minute proportion of the total nuclear chromatin. It seems unlikely, therefore, that structural histone associated with only this chromatin could comprise the major component of the missing histone fractions. More likely, most of the missing histone (20 to 40 percent of the total nuclear histone, by radioactivity) represents histone whose synthesis is coupled with the presence of the nucleolus. There is preliminary evidence that at least a portion of the nuclear histone is synthesized by the nucleolus (*11*). The results can be considered further evidence that the nucleolus is either directly or indirectly responsible for the synthesis of a large proportion of the fraction I and II histone.

How can the mutant develop and differentiate so normally in the absence of synthesis of fractions I and II? One answer to this problem may be that the increase in fractions III and IV is concommitant with the loss of the early fractions (see Fig. 1) and may indicate a transfer of function from lysine- to arginine-rich histone. Littau *et al.* (*6*) report that the arginine-rich fraction in vitro competes with the lysine-rich fraction and prevents it from clumping chromatin. Some degree of interrelationship in vivo can also be presumed. Arginine-rich histone has been reported to repress RNA synthesis and may be involved in the site-specific fine-tuning of gene readout (*12*). An amino acid analysis of *Xenopus* fractions III and IV is presently under way to determine their relationship to the arginine-rich fractions of the calf-thymus histone.

The present work represents a first step in an attempt to correlate specific histone fractions with functional and structural configurations of chromatin.

LAURENCE BERLOWITZ*
MAX L. BIRNSTIEL
Institute of Animal Genetics, University of Edinburgh, Edinburgh, Scotland

References and Notes

1. V. G. Allfrey, V. C. Littau, A. E. Mirsky, *Proc. Natl. Acad. Sci. U.S.* **49**, 414 (1963); J. Hindley, *Biochem. Biophys. Res. Commun.* **12**, 175 (1963); R. G. Huang, J. Bonner, K. Murray, *J. Mol. Biol.* **8**, 54 (1964).
2. K. Murray, *J. Mol. Biol.* **15**, 409 (1966).
3. S. W. Brown and U. Nur, *Science* **145**, 130 (1964).
4. L. Berlowitz, *Proc. Natl. Acad. Sci. U.S.* **53**, 68 (1965).
5. ——, *ibid.* **54**, 476 (1965).
6. V. C. Littau, C. J. Burdick, V. G. Allfrey, A. E. Mirsky, *ibid.* **54**, 1204 (1965); J. M. Neelin, P. X. Callahan, D. C. Lamb, K. Murray, *Can. J. Biochem. Physiol.* **42**, 1943 (1964).
7. D. D. Brown and J. B. Gurdon, *Proc. Natl. Acad. Sci. U.S.* **51**, 139 (1964).
8. H. Wallace, *J. Embryol. Exptl. Morphol.* **8**, 405 (1960); *ibid.* **10**, 212 (1962); ——— and M. L. Birnstiel, *Biochim. Biophys. Acta* **114**, 269 (1966).
9. K. Satake, P. S. Rasmussen, J. M. Luck, *J. Biol. Chem.* **235**, 2801 (1960).
10. G. R. Chalkley and H. R. Maurer, *Proc. Natl. Acad. Sci. U.S.* **54**, 498 (1965).
11. M. L. Birnstiel and G. Flamm, *Science* **145**, 1435 (19[illegible] L. S. Hnilica, M. C. Liau, R. B. Hurlbert, *ibid.* **152**, 521 (1966).
12. B. G. T. Pogo, V. G. Allfrey, A. E. Mirsky, *Proc. Natl. Acad. Sci. U.S.* **55**, 4 (1966).
13. Work carried on during the tenure of NSF science faculty fellowship 65121 (L.B.). We thank Dr. Kenneth Murray for his advice, and Ian Purdom and James Speirs for excellent technical assistance.
* Present address: Department of Biology, State University of New York, Buffalo 14214.

30 January 1967; 24 February 1967