Olins, D. E., Bryan, P. N., Harrington, R. E., Hill, W. E., & Olins, A. L. (1977) *Nucleic Acids Res. 4*, 1911.
Ong, E. C., Snell, C., & Fasman, G. D. (1976) *Biochemistry 15*, 468.
Oudet, P., Gross-Bellard, M., & Chambon, P. (1975) *Cell (Cambridge, Mass.) 4*, 281.
Parrish, J. R., & Blout, E. R. (1971) *Biopolymers 10*, 1491.
Peggion, E., Cosani, A., Turbojevich, M., & Borin, G. (1972) *Biopolymers 11*, 633.
Ramsay-Shaw, B. R., Herman, T. M., Kovacic, R. T., Beaudreau, G. S., & Van Holde, K. E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 505.
Renz, M., Nehls, P., & Hozier, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1879.
Roark, E. D., Geoghegan, T. F., & Keller, G. H. (1974) *Biochem. Biophys. Res. Commun. 59*, 542.
Ruiz-Carrillo, A., & Jorcano, J. L. (1979) *Biochemistry 18*, 760.
Schwartz, A. M., & Fasman, G. D. (1979) *Biopolymers 18*, 1045.
Simon, R. H., & Felsenfeld, G. (1979) *Nucleic Acids Res. 6*, 689.
Singer, S. J. (1962) *Adv. Protein Chem. 17*, 1.
Sperling, R., & Bustin, M. (1975) *Biochemistry 14*, 3322.
Stein, A., & Page, D. (1980) *J. Biol. Chem. 255*, 3629.
Szopa, J., Jacob, G., & Arfmann, H.-A. (1980) *Biochemistry 19*, 987.
Tanford, C. (1962) *J. Am. Chem. Soc. 84*, 4240.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 122.
Tanford, C., Buckley, C. E., De, P. K., & Lively, E. P. (1962) *J. Biol. Chem. 237*, 1168.
Thomas, G. J., Prescott, B., & Olins, D. E. (1977) *Science (Washington, D.C.) 197*, 385.
Thomas, J. O., & Kornberg, R. D. (1975) *FEBS Lett. 58*, 353.
Thomas, J. O., & Butler, P. J. G. (1977) *J. Mol. Biol. 116*, 769.
Van Holde, K. E., & Isenberg, I. (1975) *Acc. Chem. Res. 8*, 327.
Van Holde, K. E., Sahasrabuddhe, C. G., & Shaw, B. R. (1974) *Nucleic Acids Res. 1*, 1579.
Wilhelm, M. L., Mazen, A., & Wilhelm, F. X. (1977) *FEBS Lett. 79*, 404.
Woodcock, C. L. F. (1973) *J. Cell Biol. 59*, 368a.
Zama, M., Olins, D. E., Prescott, B., Thomas, G. J. (1978) *Nucleic Acids Res. 5*, 3881.

Histone Acetylation and the Deoxyribonuclease I Sensitivity of the *Tetrahymena* Ribosomal Gene†

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ABSTRACT: Under appropriate conditions, up to 85% of the total acetate can be removed from the histones of isolated *Tetrahymena* macronuclei by an endogenous histone deacetylase activity. After in vitro deacetylation, the ribosomal genes are still preferentially digested by DNase I. These observations suggested that either the majority of histone-bound acetate is unnecessary to maintain the DNase I sensitive state or ribosomal chromatin (rChromatin) histones remain acetylated under these conditions. The characteristics of histone acetylation were studied in *Tetrahymena* rChromatin, which can be isolated in a relatively pure form. Histones associated with the presumably active, DNase I sensitive ribosomal genes have a high steady-state level of histone acetylation which, surprisingly, is maintained by very low acetate turnover rates.

Most or all eucaryotic nuclear DNA, including transcriptionally active and inactive genes, is probably packaged with the four core histones (H2A, H2B, H3, and H4) into nucleosomes [for a review, see McGhee & Felsenfeld (1980)]. While the primary sequences of these histones have been highly conserved during evolution [see Isenberg (1979)], they undergo a variety of postsynthetic modifications. Since a variable fraction of potential modification sites (many of which are also highly conserved) is modified at any one time, considerable heterogeneity of nucleosomal histone may thus be generated. It is likely that this heterogeneity in some way alters the structural states of the nucleosomes, resulting in different functional states of chromatin.

The most extensively studied secondary modification of histones, acetylation, occurs at specific lysine residues near the NH_2 terminus of the four core histones [for a review, see Allfrey (1977)]. It has been postulated that this modification might weaken both histone–DNA and higher order nucleosome–nucleosome interactions, thereby making specific DNA sequences more accessible for transcription or replication (McGhee & Felsenfeld, 1980; Shewmaker & Wagner, 1980; Bode et al., 1980). Positive correlations exist between histone acetylation and transcriptional activity in a number of biological systems [see Allfrey (1977)]. Moreover, highly acetylated histones are solubilized rapidly by DNase I under conditions in which transcriptionally active chromatin is preferentially cleaved (Nelson et al., 1978a, 1979; Vidali et al., 1978; Sealy & Chalkley, 1978). Chromatin derived from cells grown in sodium butyrate, a deacetylase inhibitor (Candido et al., 1978; Boffa et al., 1978; Cousens et al., 1979), shows both an enhanced level of histone acetylation and an

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enhanced rate of digestion by DNase I (Mathis et al., 1978; Vidali et al., 1978; Nelson et al., 1978b; Simpson, 1978). While it is tempting to postulate that the increase in highly acetylated forms participates at some level in the conversion of chromatin to an activated, DNase I sensitive state, butyrate may have other, indirect effects on chromatin structure [see Candido et al. (1978), D'Anna et al. (1980), and Levy-Wilson (1981)].

So far, studies have been confined to analyses of the relationship between histone hyperacetylation and DNase I digestibility of bulk chromatin and not of specific genes. Surprisingly, while deacetylase activity has been observed in isolated nuclei (Perry et al., 1979), the effect of removal of histone acetate on the DNase I sensitivity either of bulk chromatin or of specific genes has not been determined. The relationship, therefore, between histone acetylation and transcriptional activity is circumstantial.

The core histones of *Tetrahymena* macronuclei are characterized by a high level of acetylation (Johmann & Gorovsky, 1976; Vavra et al., 1981a) which can be greatly reduced in vitro by an endogenous histone deacetylase activity (Vavra et al., 1981a; this paper). We have used this observation to compare the DNase I sensitivity of the *Tetrahymena* ribosomal gene in chromatin characterized by different degrees of histone acetylation. We report here that DNase I sensitivity of ribosomal genes in *Tetrahymena* macronuclei is unaffected by treatment of nuclei under conditions which remove 50–85% of the acetate from *bulk* histones. Since the amplified *Tetrahymena* rDNA and its associated proteins (rChromatin) can be isolated in relatively pure form, we have also been able to study histone acetylation directly in chromatin of this specific, presumably transcriptionally active, DNase I sensitive gene. Unexpectedly, our observations indicate that a high steady-state level of histone acetylation is maintained in rChromatin by very low rates of acetate turnover.

Experimental Procedures

Isotopic Labeling of Tetrahymena pyriformis Cell Cultures. (*1*) *Thymidine Labeling.* Dual-labeled macronuclei for nuclease digestions were isolated from *T. pyriformis* (an amicronucleate strain; formerly *T. pyriformis* strain GL) labeled with [^{14}C]thymidine during growth, starved, and labeled with [^{3}H]thymidine for 2 h directly after refeeding the cells, as described by Mathis & Gorovsky (1976). For some nuclease digestions, dual-thymidine-labeled cells were additionally treated as described below (see Acetate Labeling) except that [^{3}H]acetate was omitted (starved–refed, sham acetate-labeled cells). In experiments using a single thymidine label, 6.7 μCi/mL [^{3}H]thymidine was substituted for [^{14}C]thymidine, and the isotope was omitted during the refeeding period.

(*2*) *Acetate Labeling.* [^{3}H]Thymidine (single)-labeled cells were collected at room temperature at the end of the refeeding period, washed gently in $^{1}/_{3}$ volume of Tris (10 mM Tris, pH 7.4), and resuspended at their original concentration in Tris. Protein synthesis was halted by the addition of cycloheximide to 10 μg/mL (Guttman, 1978), and after 3–5 min, the cells were labeled with 25 μCi/mL sodium [^{3}H]acetate for 30 min. In pulse–chase experiments, cells were then washed once in Tris and resuspended in an equal volume of enriched proteose peptone growth medium (Gorovsky et al., 1975). At various times, cells were collected by centrifugation at 2250*g* for 1 min at 4 °C for nucleus isolation. Cells incubated for 45–60 min in [^{3}H]acetate continued to incorporate label into histone, indicating that the [^{3}H]acetate source is not exhausted during a 30-min labeling period.

(*3*) *Lysine Labeling.* "Starved–refed" cells were prepared as described above except that 16.7 μCi/mL [^{3}H]lysine was substituted for [^{14}C]thymidine during growth, 0.025 mg/mL cold thymidine replaced [^{3}H]thymidine in the refeeding medium, and unlabeled lysine was added to 0.45 mg/mL during refeeding.

Nucleus Isolation. (*1*) *Method A.* Macronuclei used for in vitro deacetylation experiments and some nuclease digestions were isolated as described by Mathis & Gorovsky (1976). Phenylmethanesulfonyl fluoride (1 mM; PMSF) was included in all isolation media.

(*2*) *Method B.* For extraction of nucleoli, pulse–chase kinetics, and some nuclease digestions, macronuclei were isolated by a modification of the procedure described by Niles (1978) to be described in detail elsewhere (M. Colavito-Shepanski and M. A. Gorovsky, unpublished experiments). Nuclei from cells lysed in the presence of Nonidet P-40 were separated from cytoplasmic contamination by pelleting through 1.8 M sucrose. Sodium butyrate (*n*-butyric acid neutralized with NaOH) at 6–30 mM and 1 mM PMSF were included in all buffers.

DNase I digestion kinetics and histone modification patterns were identical in nuclei isolated by these two methods.

In Vitro Deacetylation of Nuclei. Macronuclei were washed twice in RSB [10 mM Tris (pH 7.4)–10 mM NaCl–3 mM $MgCl_2$–1 mM PMSF] and twice in either "deacetylation medium" [10 mM EDTA (pH 8.2)–50 mM NaCl–1 mM PMSF] or "deacetylation medium plus butyrate" [10 mM EDTA (pH 8.2)–50 mM sodium butyrate–1 mM PMSF]. They were resuspended at a final concentration of 3×10^7 nuclei/mL in these deacetylation media and incubated at room temperature up to 48 h. For measurement of deacetylation kinetics, 4.5 M H_2SO_4 was added to aliquots of chromatin to a final concentration of 0.2 M and histones were extracted by method A (see Histone Extraction). For nuclease digestions, chromatin pellets were washed twice in RSB containing 50 mM butyrate and homogenized in a 0.5-mL siliconized glass homogenizer with a Teflon pestle at a concentration of 10^8 nuclei/mL. Histones were extracted from an aliquot of this suspension as described below (method A).

Nuclease Digestions. Macronuclei, chromatin, or DNA, at concentrations equivalent to about 10^8 nuclei (1.0 mg of DNA)/mL, were digested in RSB containing 50 mM butyrate. Aliquots were digested with 20–10 000 units·min/mL DNase I (Worthington) at 0 °C. For micrococcal nuclease digestions, $CaCl_2$ was added to 0.1 mM and digestion conditions of 10–1500 units·min/mL micrococcal nuclease (Sigma Chemical Co.) at 20 °C were employed. For measurement of kinetics, the digestion mixture was precipitated for 20 min in 6% Cl_3AcOH–1 M NaCl on ice, pelleted by centrifugation at 16000*g* for 10 min, and washed once, and Cl_3AcOH-precipitable material was hydrolyzed in 6% Cl_3AcOH–1 M NaCl for 2 h at 85–90 °C. The combined cold Cl_3AcOH supernatants (S) and the hot Cl_3AcOH-solubilized material (P) were counted in 10 mL of aqueous counting solution (ACS; Amersham), and the percent digestion was calculated as $S/(S + P)$. For density gradient analysis, digestions were stopped by the addition of 4 volumes of NDS [1.0% $NaDodSO_4$–0.5 M EDTA–18 mM Tris (pH 9.5)] and DNA was isolated (see DNA Isolation). Aliquots taken after Pronase digestion were Cl_3AcOH precipitated as described above [with 0.14 mg/mL bovine serum albumin (BSA) as the carrier] to measure the extent of digestion.

rChromatin Isolation. Macronuclei prepared by method B were fractionated into rChromatin and bulk components by a method to be described elsewhere (M. Colavito-Shepanski

and M. A. Gorovsky, unpublished experiments) which enriches for (rChromatin-containing) nucleoli. Nucleoli were extracted by a modification of the nuclear homogenization procedure of Gocke et al. (1978) and separated from residual nuclear contamination by density centrifugation in Percoll (Pharmacia). Preparations that are 70–82% rDNA by mass are routinely obtained with this procedure. For comparison to rChromatin, bulk nuclear chromatin samples (from which rChromatin has been extracted) were treated in a manner identical with that of rChromatin. All solutions contained 6–30 mM sodium butyrate and 1 mM PMSF.

Histone Extraction. (*1*) *Method A.* Histones were extracted as described by Allis et al. (1979) except that 3.25% perchloric acid replaced Cl_3AcOH to precipitate histones. Histone H1, which has the same mobility in acid–urea gels as tri- and tetraacetylated H4, is not precipitated by this procedure.

(*2*) *Method B.* Histones were extracted by an adaptation of the procedure of Hardy et al. (1969) to be discussed elsewhere (M. Colavito-Shepanski and M. A. Gorovsky, unpublished experiments) by incubation of chromatin in 66% acetic acid. Solubilized proteins were precipitated in cold 20% Cl_3AcOH (with BSA at 0.14 mg/mL as the carrier) after dilution with 5.6 volumes of water. Precipitated protein was processed as described by Allis et al. (1979).

Gel Electrophoresis. One-dimensional long acid–urea–acrylamide gels and two-dimensional (long acid–urea or Triton–acid–urea by $NaDodSO_4$) gels were prepared as described by Allis et al. (1980), stained as described by Vavra et al. (1981a), and fluorographed (Bonner & Laskey, 1974). Quantitation of one-dimensional gels and fluorographs is described by Vavra et al. (1981a). For quantitation of two-dimensional gels and fluorographs, successive scans were taken across each spot and the areas under the peaks summed. Total areas calculated by doubling the values obtained from half the number of sweeps did not differ by more than 5% from the values discussed here.

DNA Isolation. DNA was isolated by a modification of the procedure of Kavenoff & Zimm (1973) as described by Gorovsky & Keevert (1975). Nuclei or chromatin diluted with 4 volumes of NDS (see Nuclease Digestions) was incubated at 65 °C for 40 min. Pronase was added to a concentration of 1 mg/mL and the digestion carried out for 3–12 h at 50 °C. Digests were diluted 1:1 with water, extracted with an equal volume of chloroform–isoamyl alcohol (24:1), combined with 1 (aqueous) volume water wash of the organic phase, and precipitated overnight with 2 volumes of 95% ethanol at −20 °C. The precipitated DNA was pelleted by centrifugation at 16000*g* for 10 min, washed twice with 70% ethanol, and dried in vacuo. If necessary, nucleic acid concentrations of at least 10 μg/mL were maintained throughout this procedure by the addition of carrier yeast tRNA (Sigma).

CsCl–Hoechst Gradients. DNA isolated from undigested or slightly DNase I digested chromatin, or from rChromatin preparations, was analyzed on CsCl–Hoechst gradients as previously described (Mathis & Gorovsky, 1977).

Calculation of Histone–Acetate:DNA Ratio in Chromatin. [^{3}H]Thymidine- and [^{3}H]acetate-labeled bulk or ribosomal chromatin was fractionated by histone extraction method B to yield a "DNA" pellet and "protein" supernatant. Less than 7% of the protein fractionates with the DNA pellet, while less than 8% of the DNA remains in the supernatant (M. Colavito-Shepanski and M. A. Gorovsky, unpublished observations).

(*1*) *Determination of Histone-Bound* [^{3}*H*]*Acetate.* Before precipitation of protein, known amounts of [^{14}C]lysine-labeled bulk nuclear protein (extracted by method B from [^{14}C]-lysine-labeled nuclei) were added to each protein supernatant, and aliquots of the mixture were taken for scintillation counting. The remainder was Cl_3AcOH precipitated as described for histone extraction method B, and the resulting dried pellet was resuspended in 0.25 M sodium acetate–0.0125 M EDTA–0.05 M Tris (pH 8.0)–5 mM $NaDodSO_4$ containing 10 mg/mL Pronase. After digestion of 50 °C for 3 h, Pronase-resistant material was precipitated with 20% Cl_3AcOH, and the total amount of ^{3}H and ^{14}C in the supernatant was determined. The initial amount of protein-bound [^{3}H]acetate was derived from the fraction of ^{14}C recovered and the amount of [^{3}H]acetate released into the supernatant by Pronase.

(*2*) *DNA Determination.* Relative DNA amounts in each sample were calculated from initial [^{3}H]thymidine levels and relative DNA specific activity measurements.

The DNA pellet was washed once in 70% ethanol–0.1 M sodium acetate and once in 95% ethanol–0.1 M sodium acetate and dried in vacuo. The pellet was resuspended in 0.8% $NaDodSO_4$–0.4 M EDTA–14.4 mM Tris (pH 9.5) containing 5 mg/mL Pronase and incubated at 50 °C until cleared (3–24 h). Aliquots were Cl_3AcOH precipitated and the DNA was hydrolyzed in Cl_3AcOH at 85 °C as described for nuclease digestions. After the DNA hydrolysis, samples were precipitated on ice by addition of Cl_3AcOH to 20%. [^{3}H]Thymidine in the Cl_3AcOH-soluble supernatant was determined by scintillation counting.

The specific activity of DNA from rChromatin and bulk fractions was determined as [^{3}H]thymidine incorporation per unit of fluorescence intensity by a modification (M. Colavito-Shepanski and M. A. Gorovsky, unpublished experiments) of the fluorescence detection method of Thomas & Farquhar (1978). DNA remaining after aliquots were removed for determination of initial [^{3}H]thymidine levels (see above) was purified as described for DNA isolation. The dried pellets were resuspended in 0.3 M NaOH, and RNA was removed by alkaline hydrolysis at 37 °C for 1 h. The solution was neutralized with HCl, and the DNA was precipitated with 6% Cl_3AcOH, washed in ethanol as above, and dried. DNA (from rChromatin and bulk) was resuspended in H_2O, divided into aliquots of increasing size, and dried in vacuo. The quantity of DNA in each sample was assayed by the intensity of fluorescence produced after reaction with 3,5-diaminobenzoic acid dihydrochloride (Eastman). Aliquots of each reacted sample were taken to measure [^{3}H]thymidine content by scintillation counting. Relative specific activity was estimated by comparing the slopes of least-squares regression plots of radioactivity vs. fluorescence intensity for bulk and rChromatin.

Results

In Vitro Deacetylation of Histones by Tetrahymena Deacetylase. The normally high levels of histone acetate in isolated macronuclei can be reduced through the action of an endogenous deacetylase (Vavra et al., 1981a). When histones from nuclei incubated in deacetylation medium (in the absence of butyrate) are separated on long acid–urea–acrylamide gels, the extent of histone secondary modification is greatly reduced (Figure 1). Acetylation is responsible for most, if not all, heterogeneity in macronuclear histones H4, H3, and H2B, while the two primary sequence variants of H2A are both acetylated and phosphorylated (Johmann & Gorovsky, 1976; Allis et al., 1980). Nuclei incubated in deacetylation medium plus butyrate (see Experimental Procedures) show only a small decrease in acetylation levels, indicating that in *Tetrahymena*, as in mammalian systems, butyrate is an effective deacetylase

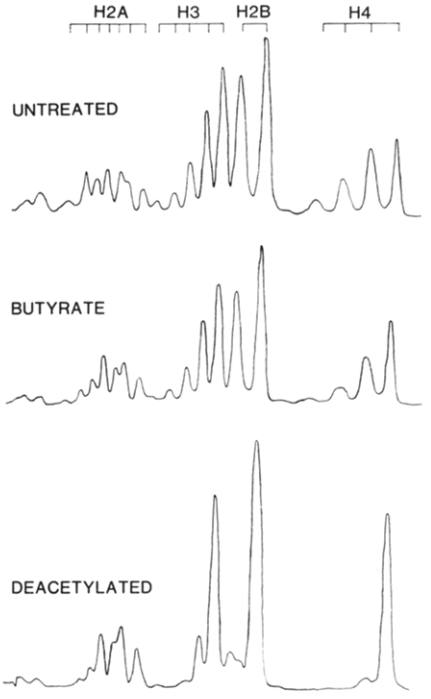

FIGURE 1: In vitro deacetylation of histones by endogenous deacetylase(s). Densitometer scans of the histone region of fast green stained long acid–urea gels. Histone modification was assayed before and after a 24-h incubation of *T. pyriformis* macronuclei in deacetylation medium in the presence or absence of 50 mM butyrate (see Experimental Procedures). Electrophoresis is from left to right, with unmodified forms having greater mobility than modified forms. Brackets indicate parental and modified forms within each histone species.

Table I: Summary of Deacetylation of Bulk Histones in Vitro[a]

% acetate remaining in[b]				
H4	H2B	H3	H2A	total
16 ± 7	43 ± 21	46 ± 14	ND	32 ± 13

[a] Determined by quantitative densitometry of fast green stained gels. Acetylation levels were calculated as the sum of protein staining at a particular level multiplied by the number of acetates per molecule at that position. [b] Values are means ± standard deviations of six experiments.

inhibitor. Quantitation of gel scans such as these yields the time course for in vitro deacetylation shown in Figure 2. The acetate in H4, H2B, and H3 is removed rapidly at first, followed by a slower rate of deacetylation. In this experiment, after 24 h, 9%, 38%, and 31% of the acetate remained in these histones, respectively. Average acetylation levels of these histones after 10–48-h incubation are shown in Table I.

It is unlikely that the slower deacetylation rates are caused solely by loss of deacetylase activity during incubation or by end-product inhibition. Washing deacetylated chromatin into fresh deacetylation medium does not lead to further deacetylation. Most of the deacetylase activity is retained in chromatin after a 24-h incubation in the presence of butyrate, as can be demonstrated by washing nuclei into fresh deacetylation medium (data not shown).

Because of its combination of acetate and phosphate modifications, the extent of histone H2A deacetylation cannot be determined by this method. In a separate experiment, nuclei isolated from cells labeled with [^{3}H]acetate for 30 min were incubated in deacetylation medium for 48 h, and the relative specific activity (unit of fluorographic intensity per unit of past green stain) of the histones was determined (Figure 3 and

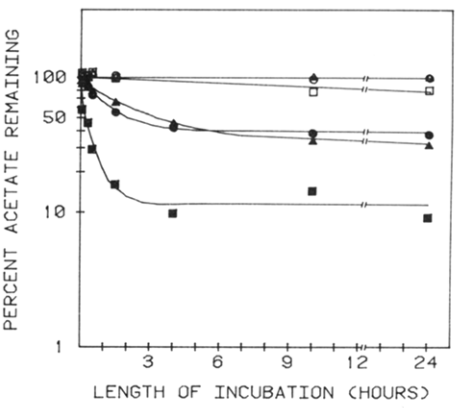

FIGURE 2: Time course of in vitro deacetylation. Histone acetylation levels were determined in macronuclei incubated 0–24 h in deacetylation medium in the presence or absence of 50 mM butyrate. The percent acetate groups remaining in each histone species was calculated by densitometric analysis and planimetry of scans of fast green stained long acid–urea gels such as those shown in Figure 1. Shown are histones H4 (□), H3 (△), and H2B (○). Solid symbols indicate deacetylated chromatin; open symbols represent the butyrate control.

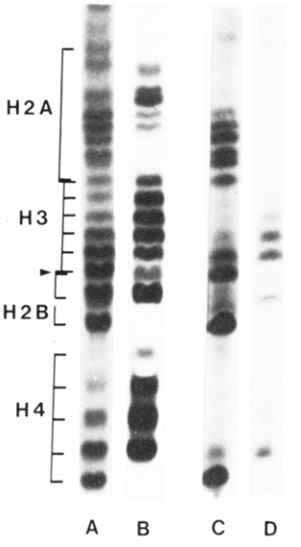

FIGURE 3: Acetylation pattern of macronuclear histones before and after incubation in deacetylation medium. [^{3}H]Acetate-labeled macronuclear histones were extracted from chromatin before (A and B) and after (C and D) a 48-h incubation in deacetylation medium and separated on long acid–urea gels. Fast green stained gels (A and C) and fluorographs (B and D) were quantitated by densitometry, and the unit fluorographic intensity per unit stain was calculated (see Table I). Brackets indicate acetylation levels within each histone species, with unmodified forms exhibiting greater mobility than modified forms. The small amount of acetate observed at the position of the unmodified form of H3 in lane B (arrowhead) is due to contributions by a small amount of diacetylated H2B and by monoacetylated hvl, an H2A-like histone variant which migrates in this region of the gel [see Allis et al. (1980) and Vavra et al. (1981a) for details of acetate labeling of hvl]. Because H2A is modified by both acetate and phosphate, the acetylation levels of H2A cannot be precisely determined. Exposure time was 4 days. No H2A-bound acetate was detectable in the deacylated chromatin in fluorographs exposed 5 times longer.

Table II). The [^{3}H]acetate pattern generated by a 30-min labeling resembles steady-state acetylation levels, although small differences in acetate distribution indicate that true steady-state labeling is not quite achieved in this time period (unpublished observations). In a single experiment (Table II, experiment 1[b]), analysis of H4, H3, and H2B by this method yielded final acetylation levels 10–15% lower than those estimated from stained gels. This discrepancy may be due to disproportionate labeling of a subset of histones which have

Table II: Histone Deacetylation in Vitro and in Vivo

expt	treatment	% acetate remaining in H4	H2B	H3	H2A	total
1[a]	in vitro deacetylation	16	20	49		33
1[b]	in vitro deacetylation	6	5	33	0	12
2[c]	in vivo pulse-chase	27	40	58	8	28

[a] Determined by quantitative densitometry of fast green stained gels. Acetylation levels were calculated as the sum of protein staining at a particular level multiplied by the number of acetates per molecule at that position. [b] Determined by quantitative densitometry of fluorographs of fast green stained gels. Acetate per unit histone was determined as the fluorographic intensity of a histone region divided by the fast green staining intensity. [c] Determined by quantitative densitometry of fluorographs of acetate-labeled histones isolated from known amounts of chromatin.

rapid turnover rates or to the existence of a subset of acetylated histones which have little or no acetate turnover. All detectable H2A-associated [^{3}H]acetate labeled in a 30-min pulse is removed in deacetylated chromatin. Since the [^{3}H]acetate level of H2A measured this way is lower than that of H4, it is likely that greater than 90% of total H2A–acetate has been removed in this in vitro deacetylation. While final acetylation levels vary from preparation to preparation, 50–85% of the total histone–acetate can routinely be removed during a 10–48-h incubation.

The different histones appear to be deacetylated at different rates and to different extents during incubation in vitro. Histone H4 is deacetylated more rapidly than the other histones, while histones H2B and H3 are deacetylated at a relatively slow rate. H2A is most likely deacetylated at a rapid rate, in view of its essentially complete deacetylation. To test whether these rate differences reflect in vivo differences, we incubated cells with [^{3}H]acetate for 30 min and subsequently chased the label during a 40-min incubation in growth medium. [^{3}H]Acetate decreased in histones H4 and H2A rapidly while H2B- and H3-associated acetate turned over more slowly (Table II, experiment 2). Moreover, total histone-bound [^{3}H]acetate decreased during this chase in a manner similar to in vitro deacetylation kinetics, displaying both rapid and slow turnover rates (Moore et al., 1979; Vavra et al., 1981a; unpublished observations).

A portion of the remaining acetylated subspecies observed after in vitro incubation may reflect a real limit imposed by a subset of acetylated molecules which have no acetate turnover. Another alternative is that acetate from a fraction of each histone species is completely removed by rapid deacetylation, while a fraction with a slow turnover rate is only partially deacetylated under these conditions. As can be seen in Tables I and II and Figure 2, the more rapid the initial deacetylation rate for a particular histone (both in vitro and in vivo), the lower the final acetylation level in deacetylated chromatin. Moreover, the histone species seem to contain different amounts of the rapidly and slowly turning over components; most acetate on histone H4 appears to turn over rapidly, while H3 and H2B must contain a greater proportion of molecules whose acetate turns over at slower rates (Vavra et al., 1981a). These results suggest that most of the histones characterized by rapid acetate turnover are deacetylated after this treatment, while a variable, probably moderate amount of acetate has been released from histones in the slower kinetic population. However, detailed kinetic analyses of acetate release in vitro after labeling in vivo for various periods will be necessary to establish the precise relationship between deacetylation rates in vivo and in vitro.

Histone Deacetylation and DNase I Sensitivity of rChromatin. The ribosomal genes (rDNA) in *Tetrahymena* are extrachromosomal, amplified sequences comprising about 2% of the macronuclear DNA [for a review, see Gorovsky (1980)]. They can be preferentially labeled by using a defined starvation–refeeding protocol to yield nuclei in which bulk DNA has been labeled with [^{14}C]thymidine during exponential growth and rDNA has preferentially incorporated [^{3}H]thymidine during a 2-h refeeding period (Engberg et al., 1974; Mathis & Gorovsky, 1976; Piper et al., 1976). rRNA is synthesized in these cells (Nilsson & Leick, 1970).

It has been shown previously that DNase I preferentially cleaves the ribosomal gene in intact nuclei from starved–refed cells (Mathis & Gorovsky, 1977; Vavra & Gorovsky, 1978; Giri & Gorovsky, 1980). We have calculated that most or all of the rDNA must be digested to small fragments about 4–5 times faster than bulk inactive chromatin (Vavra & Gorovsky, 1978; Vavra et al., 1981b), a rate similar to that of most active genes (Garel et al., 1977). Because the preferentially labeled rDNA is also preferentially digested in cells "chased" into exponential growth (Mathis & Gorovsky, 1977) and since genes appear to become DNase I sensitive very rapidly after replication (Weintraub, 1979), the preferential cleavage we observe probably is not due to some property of newly replicated chromatin but rather to the structure typical of an active gene.

Figure 4 shows kinetics of DNase I and micrococcal nuclease digestions of dual-labeled nuclei. The digestion of ^{3}H-labeled (rDNA-enriched) DNA is plotted vs. that of ^{14}C-labeled (bulk) DNA so that, if both labels were released at equal rates, digestion points would fall on a 45° straight line (shown in Figure 4 for reference). This method of analyzing digestion kinetics allows us to compare directly many independent sets of digestions at once, because only relative, not absolute, digestion rates are involved. That ^{3}H-labeled (rDNA-enriched) chromatin is reduced to Cl_3AcOH-soluble fragments faster than ^{14}C-labeled macronuclear chromatin by DNase I can be seen in Figure 4A. Figure 4B shows kinetics of digestion by micrococcal nuclease. The [^{3}H]chromatin is digested more slowly than ^{14}C-labeled bulk macronuclear chromatin, possibly due to the A-T preference of micrococcal nuclease (Roberts et al., 1962; Simpson & Kunzler, 1979; Bryan et al., 1979); rDNA has a higher G-C content than bulk DNA (Allen & Gibson, 1973; Karrer, 1976). This effect can be seen in the digestion of purified DNA by this nuclease as well (Figure 4B).

To test the effect of histone deacetylation on the nuclease sensitivity of an active gene, we have incubated these dual-labeled nuclei in deacetylation medium in the presence or absence of sodium butyrate and digested the resulting chromatin with DNase I or micrococcal nuclease. In chromatin from which 85% of the total histone–acetate has been removed, ^{3}H-labeled (rDNA-enriched) material is preferentially cleaved by DNase I to the same degree as observed in intact nuclei or in chromatin which had been incubated under deacetylation conditions in the presence of butyrate (Figure 4A).

We have confirmed that the ribosomal gene containing chromatin (rChromatin) is, in fact, digested more rapidly by DNase I. CsCl–Hoechst density gradient analysis of DNA isolated from briefly DNase I digested chromatin (Figure 5) demonstrates that rDNA disappears more rapidly than bulk DNA in both deacetylated and "butyrate-control" chromatin.

Incubation of nuclei in deacetylation buffer has no effect on the relative micrococcal nuclease digestion kinetics (Figure

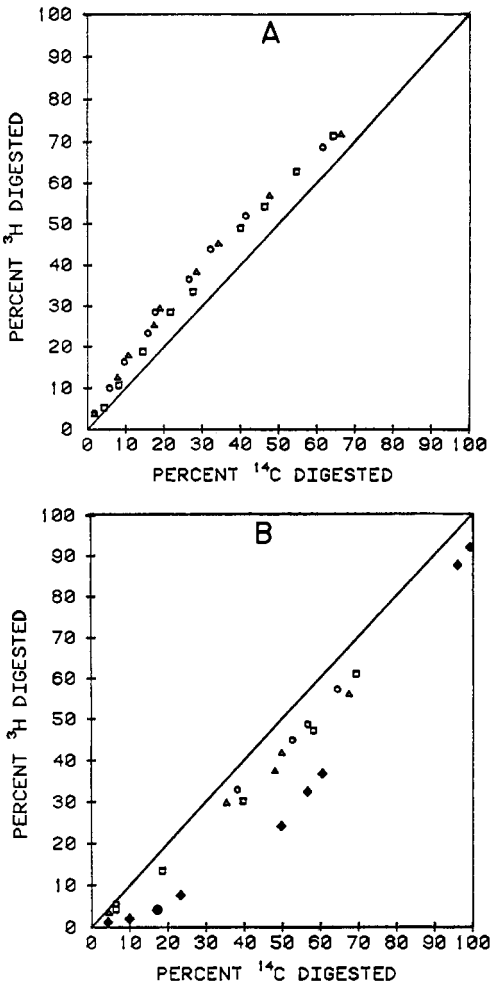

FIGURE 4: DNase I and micrococcal nuclease digestion kinetics of untreated, deacetylated, and control, butyrate-treated chromatin. (A) Starved–refed dual-labeled nuclei were incubated in deacetylation medium in the presence (▲) or absence (○) of 50 mM butyrate for 48 h at 22 °C and then washed and digested in RSB with DNase I (see Experimental Procedures). Digestion kinetics of intact, untreated nuclei are also shown (□). In starved–refed nuclei, bulk DNA is labeled with [^{14}C]thymidine, and [^{3}H]thymidine-labeled DNA is 50–70% rDNA. Since the ^{3}H-labeled DNA is not pure for rDNA and macronuclear chromatin contains a substantial DNase I sensitive component (Vavra et al., 1981b) and because nuclease digestions do not strictly obey zero-order or first-order kinetics, the relative rates of digestion of rChromatin and bulk DNase I insensitive chromatin have been obtained by using a computerized curve fitting analysis (Vavra et al., 1981b). In this experiment, deacetylated chromatin (○) was found to be depleted of 85% of the original histone acetate. Digestion was measured as the production of Cl_3AcOH-soluble material. (B) Untreated nuclei (□), deacetylated (○), and control, butyrate-treated chromatin (▲), prepared as above, were digested with micrococcal nuclease in RSB containing 0.1 mM $CaCl_2$. Digestion of free DNA from starved–refed nuclei is also shown (◆).

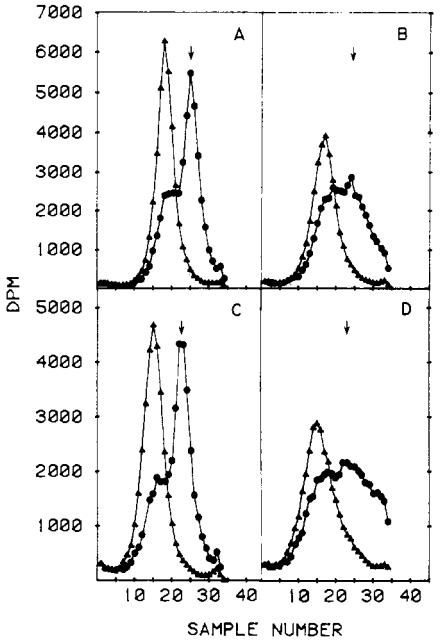

FIGURE 5: Density gradient analysis of DNA from DNase I digested deacetylated starved–refed chromatin. CsCl–Hoechst gradients were used to separate rDNA from bulk DNA in undigested and briefly DNase I digested chromatin. Bulk DNA is labeled with [^{14}C]thymidine (▲). [^{3}H]Thymidine-labeled material (●) is 66% rDNA in undigested nuclear DNA. Fraction 1 is at the top of the gradient and the rDNA position is shown (↓). (A) DNA from undigested deacetylated chromatin; (B) DNA from deacetylated chromatin digested to about 2% (^{3}H) and 1% (^{14}C) Cl_3AcOH soluble with DNase I; (C) DNA from undigested control, butyrate-treated chromatin; (D) DNA from butyrate-treated chromatin digested to about 2% (^{3}H) and 1% (^{14}C) Cl_3AcOH soluble with DNase I.

4B). Thus, it is clear that incubation of nuclei in deacetylation buffer does not induce a general artifactual sensitivity of the ^{3}H-labeled chromatin to nuclease. We conclude that removal of up to 85% of histone acetate from bulk chromatin does not affect the activated, DNase I sensitive structure of the ribosomal gene.

Histone–Acetate Levels and Turnover Rates in rChromatin. Failure of deacetylation of bulk chromatin to affect the DNase I sensitivity of ribosomal genes has two possible explanations. Either high levels of histone–acetate are not crucial to the maintenance of the DNase I sensitive state in rChromatin or rChromatin remains largely acetylated under conditions where bulk chromatin is deacetylated. The latter situation could arise either from extremely high initial acetate levels or from low rates of histone deacetylation in rChromatin.

Because the ribosomal genes are amplified, extrachromosomal, and associated with nucleoli, it is possible to isolate the chromatin associated with these activated genes in relatively pure form. However, we have not succeeded in extracting nucleoli from nuclei treated with deacetylation medium, and deacetylase activity decreases dramatically in chromatin homogenized in the nucleolus extraction buffer. Therefore, it has not been possible to measure directly the acetate levels in isolated rChromatin after in vitro deacetylation. However, a comparison of in vivo steady-state acetylation levels and acetate turnover rates in bulk chromatin and rChromatin should indicate whether the final acetylation levels in bulk and ribosomal gene containing chromatin after in vitro deacetylation are likely to be similar.

To determine the steady-state acetylation levels of rChromatin histones, we examined by two-dimensional (long acid–urea by $NaDodSO_4$) gel electrophoresis the histones associated with isolated nucleoli. In these and subsequent comparisons of bulk and rChromatin histones, it is important to note that the bulk histones are isolated from the nuclear chromatin from which rChromatin has been extracted, and thus, both chromatin fractions are subjected to similar conditions throughout the fractionation procedure. Typically 75–80% of the DNA in our nucleolar preparations is rDNA. Studies to be published elsewhere demonstrate that the histone:DNA ratio for each of the core histones is similar in bulk and rChromatin (M. Colavito-Shepanski and M. A. Gorovsky, unpublished observations). Figure 6 shows fluorographs of lysine-labeled acid-extracted proteins from bulk nuclear chromatin (Figure 6A) and from ribosomal chromatin (Figure 6B). Preparations of ribosomal chromatin are contaminated with nucleolar nonhistone proteins, some of which migrate in two-dimensional

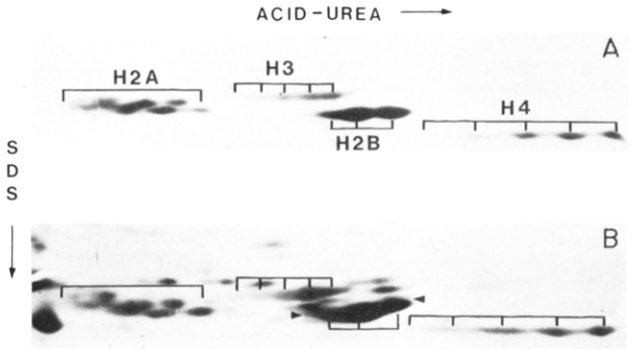

FIGURE 6: Steady-state acetylation levels of histones in bulk macronuclear and ribosomal chromatin. Lysine-labeled nuclei were fractionated into nucleoli and bulk chromatin, and proteins extracted from these fractions were separated by two-dimensional [long acid–urea by $NaDodSO_4$ (SDS in the figure)] gel electrophoresis. Fluorographs of bulk chromatin proteins (A) and nucleolar (rChromatin) proteins (B) are shown. Brackets indicate the modification levels within each histone species, with unmodified forms having a greater mobility in the acid–urea dimension than do modified forms. rChromatin preparations are contaminated by nonhistone nucleolar proteins, some of which have mobilities similar to those of the histones. The H4 and H3 regions of the rChromatin fluorograph (B) are relatively free of nonhistone contaminants, while H2B is probably slightly contaminated by nonhistone proteins in the regions marked by arrowheads. Quantitative densitometry of fluorographs such as these yields acetate distributions for H4, H3, and H2B, as shown in Figure 7. The two primary sequence variants of H2A, which differ in their mobility in $NaDodSO_4$ gels, are both acetylated and phosphorylated, so quantitation of H2A–acetate from these fluorographs is not possible. It is apparent in the figure, however, that substantial differences in modification pattern of H2A probably do not exist between bulk and rChromatin.

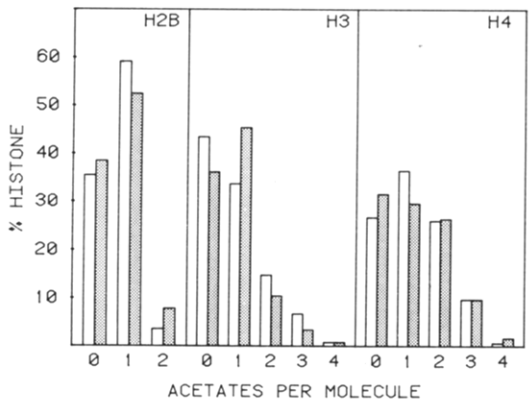

FIGURE 7: Distribution of histone–acetate in ribosomal and bulk chromatin. Fluorographs such as those shown in Figure 6 were quantitated by microdensitometry (see Experimental Procedures), and the percent of histone at each modification level was calculated for H2B, H3, and H4. Open bars = bulk chromatin; shaded bars = rChromatin. The acetate distribution of rChromatin H2B shown here probably includes contributions by nonhistone proteins (see arrowheads in Figure 6B). Since contaminants appear to contribute to the parental (zero acetates) and second-modified (two acetates) forms of H2B, the distribution of acetate in rChromatin-associated H2B is probably even more similar to the bulk distribution than indicated by our measurements.

gels with mobilities similar to those of the histones. The complex H2A pattern seen in Figure 6 is a result of two primary H2A sequence variants modified by both phosphorylation and acetylation and is additionally complicated by comigration of nonhistone proteins. The H2B region may be slightly contaminated by nonhistone proteins as well, while H3 and H4 appear to be relatively free of such contamination. The distribution of modified forms (determined by densitometry) of H2B, H3, and H4 in the two preparations is shown in Figure

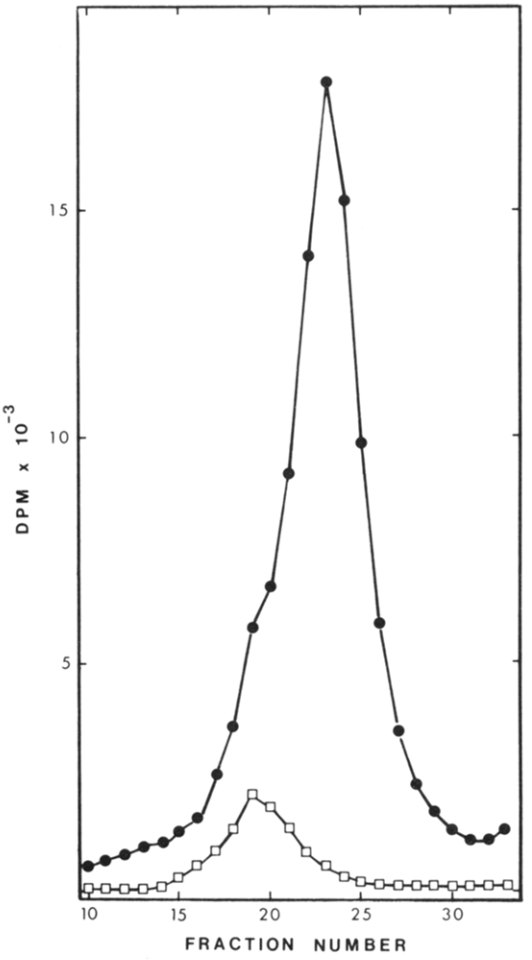

FIGURE 8: CsCl–Hoechst density gradient analysis of DNA from isolated ribosomal chromatin. [^{3}H]Thymidine- and [^{3}H]acetate-labeled nuclei were fractionated to yield rChromatin and bulk chromatin. DNA was isolated from an aliquot of the rChromatin preparation and examined by CsCl–Hoechst density gradient analysis. [^{14}C]-Thymidine-labeled bulk DNA was included in the gradients. Direction of sedimentation is from left to right. (●) [^{3}H]Thymidine-labeled rChromatin DNA; (□) ^{14}C-labeled bulk marker DNA. The percent rDNA in the rChromatin preparation (78%) was determined from the areas formed by dropping a line perpendicular to the base line at the inflection point on the bulk side of the rChromatin DNA peak. Areas were corrected for DNA specific activity differences in bulk and rDNA.

7. It is clear that the steady-state acetylation levels of these three rChromatin histones are similar to those of bulk histones and that none of the core histones differs extensively in its modification pattern from that of bulk. Thus, rChromatin is unlikely to remain highly acetylated in in vitro deacetylated preparations simply due to a higher initial level of histone-bound acetate.

The steady-state acetylation levels of histones in rChromatin could be maintained by various acetate turnover rates; if rChromatin histones remain acetylated in deacetylated chromatin due to a lack of deacetylase activity, then a comparable low-level acetylation rate would be predicted. For comparison of the acetylation rates of rChromatin histones to those of bulk histones, nuclei were isolated from cells labeled in log phase growth with [^{3}H]thymidine and subsequently labeled for 30 min with [^{3}H]acetate (see Experimental Procedures). Because the only significant acetate-labeled protein component in nuclear and nucleolar preparations is histone (Allis et al., 1980; Vavra et al., 1981a; unpublished observations), and the histone:DNA ratio is similar in bulk and ribosomal chromatin (M. Colavito-Shepanski and M. A. Gorovsky, unpublished

observations), we can calculate relative histone–acetate specific activities from the [^{3}H]acetate:DNA ratio. In an rChromatin preparation estimated by CsCl–Hoechst density gradient analysis to be 78% rDNA (Figure 8), the histone specific activity was found to be 35% that of bulk. Assuming that the remaining 22% of the chromatin has a [^{3}H]acetate:DNA ratio like that of bulk chromatin, we calculate that pure rChromatin histones have incorporated [^{3}H]acetate at a rate only 17% that of bulk. This experiment has been repeated 3 times with similar results. It appears, therefore, that under these conditions, rChromatin exhibits low levels of histone acetylase activity. Since the steady-state level of histone acetylation in rChromatin is moderately high (Figure 7), this low level of acetate incorporation *must* be matched by a comparably low level of histone deacetylase activity. We think it likely, therefore, that under our in vitro deacetylation conditions where an endogenous activity has removed 50–85% of bulk chromatin histone-bound acetate, rChromatin histones retain their steady-state acetylation levels.

Discussion

Steady-State Acetylation Levels and DNase I Sensitivity in Ribosomal Chromatin. We have shown in this report that ribosomal chromatin exhibits a high steady-state acetylation level. However, this level does not differ significantly from the normally high steady-state levels of bulk macronuclear chromatin. Since most or all of the ribosomal chromatin is probably DNase I sensitive, in contrast to about 30% of bulk chromatin (Vavra & Gorovsky, 1978; Vavra et al., 1981b), high steady-state acetate levels cannot be directly correlated with active gene structure at the same level as is probed by this nuclease. They might, however, contribute to its structure at a more fundamental level necessary for the establishment (but not necessarily the maintenance) of the DNase I sensitive state. Alternatively, high levels of acetylated histones may be necessary but not sufficient for DNase I sensitivity.

Acetate Turnover and the DNase I Sensitive State in Ribosomal Chromatin. Several populations of histones can be distinguished in chromatin on the basis of their rates of acetylation and deacetylation. Studies in a variety of systems have determined that one population of histones is modified very rapidly, while another population (usually comprising the majority of histones) is acetylated and deacetylated at a slow rate (Moore et al., 1979; Covault & Chalkley, 1980; Vavra et al., 1981a). There also appears to be a subset of histones which cannot be acetylated. Additionally, since prolonged incubation of mammalian cells in butyrate results in the bulk histone pattern containing partially acetylated histones even after almost all of the radioactive acetate has become associated with high modification levels, we cannot rule out the possibility that a class of histones containing a variety of acetylation levels exists which, once acetylated, has no appreciable acetate turnover. Thus, a wide range of histone acetylation levels can be maintained in chromatin by a variety of very different turnover rates.

Alterations in histone acetylation level which appear to be related to changes in transcriptional activity may be controlled at the level of either (or both) deacetylase activity (Pogo et al., 1968; Libby, 1970; Boffa et al., 1971; Sanders et al., 1973) or acetylase activity (Vavra et al., 1981a). Because alterations and maintenance of steady-state acetylation patterns appear to be modulated in a complex fashion at several distinct levels by histone acetylases and deacetylases, any analysis of the importance of histone acetylation in the structure of an active gene must necessarily consider not only its steady-state acetate level but also the turnover rates maintaining that level.

The acetate incorporation rate of rChromatin histones measured here is less than 20% that of bulk histones. Because a substantial fraction of rChromatin histones is acetylated at one or more sites, most of this acetate must turn over very slowly. If rapid acetate turnover exists at all in rChromatin, it must necesssarily be occurring on a *very* small fraction of histones.

rChromatin exhibits a DNase I sensitivity which appears to be typical of transcriptionally active genes. Thus, a large flux of acetate does not seem to be necessary for maintaining the "activated" chromatin structure detected by DNase I in this gene. In addition, if a very small fraction of rChromatin histones were, in fact, turning over acetate rapidly, it is unlikely that DNase I sensitivity could depend upon them, since in vitro deacetylation of chromatin-bound histones probably removes at least 50–85% of this acetate. Because rDNA fully retains its sensitivity to DNase I even after in vitro deacetylation, these acetates must not be necessary for maintenance of the DNase I sensitive state. If maintenance of this state in the ribosomal gene is contingent upon any aspect of histone acetylation, it must be upon a population of acetylated histones which has very slow acetate turnover.

While rChromatin exhibits little acetate turnover, its steady-state acetylation levels are indistinguishable from those of bulk. Clearly, the histones associated with rDNA must acquire their acetate in some fashion. Two extreme models can be considered: low net turnover rates can be achieved by a very slow constant turnover of acetate or by a brief, rapid acetylation event without subsequent turnover. It is tempting to hypothesize that such a rapid event might occur during or very shortly after replication [as does establishment of DNase I sensitivity; see Weintraub (1979)] and that all further turnover is then halted in these activated genes. We estimate that no more than about 15% of the rDNA can be replicated during the 30-min [^{3}H]acetate labeling period [see Engberg et al. (1974)]. While our value for the rChromatin acetate incorporation level (~15% of bulk) could, therefore, correspond to the acetylation of this newly replicated chromatin, uncertainty in the purity estimates of our rChromatin preparations as well as the existence of different populations of histones labeling at different rates in bulk chromatin makes it impossible to distinguish between this possibility and a slow, steady turnover rate.

If histone pool sizes in *Tetrahymena* are small, DNA synthesized in the absence of continued protein synthesis may be lacking histone. Since rDNA is probably replicated more rapidly than bulk DNA during the labeling period, an artifactually low rChromatin histone specific activity could be measured by our method. We think it unlikely that this effect is responsible for the reduced acetate incorporation level for the following reasons. Histone synthesis is not tightly coupled to DNA synthesis in starved–refed cells, which exhibit a noncoordinate synthesis of individual histones. This offers the possibility that sizable histone pools exist in these cells (Mathis, 1977). Moreover, an estimated maximum of 15% rDNA synthesis during this 30-min time period could not explain an observed 6-fold reduction in the acetate:DNA ratio. In fact, rChromatin isolated from cells pulse labeled with [^{3}H]acetate for 5 min (8-min total cycloheximide exposure) showed only 5% of the acetate:DNA ratio of bulk (data not shown). Thus, while some DNA synthesized during exposure to cycloheximide may not become associated with histones in a normal fashion (Weintraub, 1973; Seale & Simpson, 1975), its contribution to our estimates of histone specific activity is probably negligible.

Is Ribosomal Chromatin Unique? Ribosomal genes are transcribed by a special polymerase [polymerase I; see Roeder (1976) for a review] in the unique environment of the nucleolus. In *Tetrahymena* macronuclei, the genes are small, extrachromosomal palindromic dimers and so may possess a higher order structure different from that of bulk chromosomal DNA. Thus, control of many aspects of ribosomal gene transcription in *Tetrahymena* may have evolved under a set of selective pressures different from those of most active genes. rChromatin has both similarities to and differences from active nonribosomal chromatin. It has a histone:DNA ratio (M. Colavito-Shepanski and M. A. Gorovsky, unpublished observations) and micrococcal nuclease repeat pattern (Mathis & Gorovsky, 1976; Piper et al., 1976) similar to those of bulk, and it is cleaved preferentially by DNase I at rates comparable to those of other active genes (Vavra & Gorovsky, 1978; Giri & Gorovsky, 1980; Garel et al., 1977; Vavra et al., 1981b). Yet, electron-microscopic visualization of transcriptionally active ribosomal genes usually reveals a smooth, extended morphology, in contrast to the beaded structure observed for most active nonribosomal transcription units [see Mathis et al. (1980) for a review]. These morphological differences may reflect the fact that nucleosomes unfold and remain unfolded on active ribosomal chromatin but fold and unfold rapidly on active nonribosomal chromatin [see Vavra et al. (1982)]. As ribosomal chromatin and nonribosomal chromatin share many common structural features, this difference could represent a subtle alteration in the acetate metabolism of otherwise similar nucleosomes. Alternatively, the slow turnover rate of histone acetate in *Tetrahymena* rChromatin could be associated with some other unique feature of ribosomal genes. Clearly, the precise relationships between the acetylation levels of specific histones and their acetate turnover rates on the one hand and DNase I sensitivity and the regulation of transcriptional activity on the other remain to be elucidated.

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References

Allen, S. L., & Gibson, I. (1973) in *Biology of Tetrahymena* (Elliott, A. M., Ed.) pp 307–373, Dowden, Hutchenson and Ross, Stroudsburg, PA.

Allfrey, V. G. (1977) in *Chromatin and Chromosome Structure* (Li, H. J., & Eckhardt, R. A., Eds.) pp 167–191, Academic Press, New York.

Allis, C. D., Glover, C. V. C., & Gorovsky, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4857–4861.

Allis, C. D., Glover, C. V. C., Bowen, J. K., & Gorovsky, M. A. (1980) *Cell (Cambridge, Mass.) 20*, 609–617.

Bode, J., Henco, K., & Wingender, E. (1980) *Eur. J. Biochem. 110*, 143–152.

Boffa, L. C., Gershey, E. L., & Vidali, G. (1971) *Biochim. Biophys. Acta 254*, 135–143.

Boffa, L. C., Vidali, G., Mann, R. S., & Allfrey, V. G. (1978) *J. Biol. Chem. 253*, 3364–3366.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Bryan, P. N., Wright, E. B., & Olins, D. E. (1979) *Nucleic Acids Res. 6*, 1449–1465.

Candido, E. P. M., Reeves, R., & Davie, J. R. (1978) *Cell (Cambridge, Mass.) 14*, 105–113.

Cousens, L. S., Gallwitz, D., & Alberts, B. M. (1979) *J. Biol. Chem. 254*, 1716–1723.

Covault, J., & Chalkley, R. (1980) *J. Biol. Chem. 255*, 9110–9116.

D'Anna, J. A., Tobey, R. A., & Gurley, L. R. (1980) *Biochemistry 19*, 2656–2671.

Engberg, J., Nilsson, J. R., Pearlman, R. E., & Leick, V. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 894–898.

Garel, A., Zolan, M., & Axel, R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4867–4871.

Giri, C. P., & Gorovsky, M. A. (1980) *Nucleic Acids Res. 8*, 197–214.

Gocke, E., Leer, J. C., Nielsen, O. F., & Westergaard, O. (1978) *Nucleic Acids Res. 5*, 3993–4006.

Gorovsky, M. A. (1980) *Annu. Rev. Genet. 14*, 203–239.

Gorovsky, M. A., & Keevert, J. B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3536–3540.

Gorovsky, M. A., Yao, M.-C., Keevert, J. B., & Pleger, G. L. (1975) *Methods Cell Biol. 9*, 311–327.

Guttman, S. G. (1978) Ph.D. Thesis, University of Rochester, Rochester, NY.

Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mora, G. (1969) *Biochemistry 8*, 2897–2905.

Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159–191.

Johmann, C. A., & Gorovsky, M. A. (1976) *Arch. Biochem. Biophys. 175*, 694–699.

Karrer, K. (1976) Ph.D. Thesis, Yale University, New Haven, CT.

Kavenoff, R., & Zimm, B. H. (1973) *Chromosoma 41*, 1–27.

Levy-Wilson, B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2189–2193.

Libby, P. R. (1970) *Biochim. Biophys. Acta 213*, 234–236.

Mathis, D. J. (1977) Ph.D. Thesis, University of Rochester, Rochester, NY.

Mathis, D. J., & Gorovsky, M. A. (1976) *Biochemistry 15*, 750–755.

Mathis, D. J., & Gorovsky, M. A. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 773–778.

Mathis, D. J., Oudet, P., Wasylyk, B., & Chambon, P. (1978) *Nucleic Acids Res. 5*, 3523–3547.

Mathis, D., Oudet, P., & Chambon, P. (1980) *Prog. Nucleic Acid Res. Mol. Biol. 24*, 1–55.

McGhee, J., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115–1156.

Moore, M., Jackson, V., Sealy, L., & Chalkley, R. (1979) *Biochim. Biophys. Acta 561*, 248–260.

Nelson, D. A., Perry, W. M., & Chalkley, R. (1978a) *Biochem. Biophys. Res. Commun. 82*, 356–363.

Nelson, D. A., Perry, M., Sealy, L., & Chalkley, R. (1978b) *Biochem. Biophys. Res. Commun. 82*, 1346–1353.

Nelson, D., Perry, M. E., & Chalkley, R. (1979) *Nucleic Acids Res. 6*, 561–574.

Niles, E. G. (1978) *Biochemistry 17*, 4839–4844.

Nilsson, J. R., & Leick, V. (1970) *Exp. Cell Res. 60*, 361–372.

Perry, M., Nelson, D., Moore, M., & Chalkley, R. (1979) *Biochim. Biophys. Acta 561*, 517–525.

Piper, P. W., Celis, J., Kaltoft, K., Leer, J. C., Nielsen, O. F., & Westergaard, O. (1976) *Nucleic Acids Res. 3*, 493–506.

Pogo, B. G. T., Pogo, A. O., Allfrey, V. G., & Mirsky, R. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 1337–1344.

Roberts, W. K., Dekker, C. A., Rushizky, G. W., & Knight, C. A. (1962) *Biochim. Biophys. Acta 55*, 664–673.

Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 285–329, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanders, L. A., Schechter, N. M., & McCarty, K. S. (1973) *Biochemistry 12*, 783–791.
Seale, R. L., & Simpson, R. T. (1975) *J. Mol. Biol. 94*, 479–501.
Sealy, L., & Chalkley, R. (1978) *Nucleic Acids Res. 5*, 1863–1876.
Shewmaker, C. K., & Wagner, T. E. (1980) *Eur. J. Biochem. 107*, 505–510.
Simpson, R. T. (1978) *Cell (Cambridge, Mass.) 13*, 691–699.
Simpson, R. T., & Kunzler, P. (1979) *Nucleic Acids Res. 6*, 1387–1415.
Thomas, P. S., & Farquhar, M. N. (1978) *Anal. Biochem. 89*, 35–44.
Vavra, K. J., & Gorovsky, M. A. (1978) *J. Cell Biol. 79*, 126a.
Vavra, K. J., Allis, C. D., & Gorovsky, M. A. (1981a) *J. Biol. Chem.* (in press).
Vavra, K. J., Pederson, D., & Gorovsky, M. A. (1981b) *Nucleic Acids Res. 9*, 5825–5843.
Vavra, K. J., Colavito-Shepanski, M., & Gorovsky, M. A. (1982) *Cell Nucl.* (in press).
Vidali, G., Boffa, L. C., Bradbury, E. M., & Allfrey, V. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2239–2243.
Weintraub, H. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 247–256.
Weintraub, H. (1979) *Nucleic Acids Res. 7*, 781–792.

Effects of Highly Purified Estrogen Receptors on Gene Transcription in Isolated Nuclei†

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ABSTRACT: This report describes the direct effect of a highly purified hen oviduct estrogen receptor on the rates of RNA polymerase II dependent gene transcription in isolated nuclei. When this receptor was added to an in vitro transcription system containing oviduct nuclei from chronically estrogen-treated chicks withdrawn from estrogen for 0, 24, 36, 48, 60, or 72 h, the rates of incorporation of [^{3}H]UMP into RNA were increased by 1.4–3.8-fold. This effect was dependent upon receptor concentration, being half-maximal at 0.25 nM, and was inhibited by the addition of either α-amanitin (1 μg/mL) or actinomycin D (40 μg/mL). The increase in transcription rates was not demonstrable with spleen or erythrocyte nuclei, and increases in polymerase II activity in vitro were not observed with estrogen alone. These results demonstrate that the increases in RNA polymerase II activity observed following estrogen administered in vivo are mediated by the action of estrogen–receptor complexes on oviduct nuclei. The synthesis of new RNA polymerase II is not essential. When the RNA synthesized in vitro was isolated and assayed for products of ovalbumin gene expression, the nuclei from chronically stimulated and 24-h estrogen-withdrawn oviducts showed 1.6–1.8-fold increases in ovalbumin gene expression in the presence of the highly purified receptor. Thus, within 24 h of estrogen withdrawal, exogenous receptor–estradiol complexes can mimic the effect of estrogen administered in vivo on ovalbumin mRNA synthesis. After 36 and 60 h of estrogen withdrawal, the addition of receptor did not produce increases in ovalbumin gene expression despite increases in RNA polymerase II activity. These results imply that this particular estrogen receptor requires other estrogen-dependent factors before it can directly cause specific initiation of the ovalbumin gene in nuclei isolated after 24 h of hormonal withdrawal.

The relationship between nuclear estrogen receptors and egg white protein gene transcription in vivo (Tsai et al., 1975; Mulvihill & Palmiter, 1977) has been recently confirmed and extended by using an in vitro transcription assay (Taylor et al., 1980; Smith & Taylor, 1981). This system utilizes relatively short pulses of [^{3}H]UTP labeling, allowing the detection of RNA molecules synthesized de novo. It is minimally affected by the rate of RNA degradation, a factor which is also hormonally modified (Palmiter & Carey, 1974). The optimization and validation of this assay, therefore, have provided an important tool for the investigation of class II transcriptional regulation under readily manipulated cell-free conditions.

It is our ultimate goal to characterize, purify, and examine in vitro the role of the particular chick oviduct proteins which confer the specificity of estrogenic responses at the genomic level. We have identified and characterized three distinct estrogen binding proteins in chick oviduct tissue, X, Y, and Z (Smith et al., 1979; Taylor & Smith, 1982), and have highly purified the higher affinity nuclear X receptor (Taylor & Smith, 1979; Smith & Schwartz, 1979).

To investigate the effect of addition of pure estrogen receptors on gene transcription in isolated nuclei, it was preferable to first validate that our cell-free system was capable of faithful transcription. In particular, it was pertinent to determine whether the extent of transcription in vitro was dependent upon the concentration of nuclear estrogen receptors. Validation of these parameters was achieved by the withdrawal and secondary stimulation in vivo of estrogen-treated chicks. These studies documented a highly significant relationship between nuclear acceptor site occupancy and

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