Histone H4 Acetylation and Transcription in Amphibian Chromatin

John Sommerville, Jennifer Baird, and Bryan M. Turner*

School of Biological and Medical Sciences, University of St. Andrews, St. Andrews, Fife, KY16 9TS, Scotland; and *Department of Anatomy, University of Birmingham Medical School, Birmingham B15 2TJ, England

Abstract. Lampbrush chromosomes from oocytes of the amphibian Triturus cristatus have been used to examine the role of histone acetylation in transcription by indirect immunofluorescence with antisera to H4 acetylated at specific lysine residues. Electrophoresis on acid-urea-Triton gels and Western blotting have confirmed the specificity of these antisera and defined the order in which particular lysine residues are acetylated in amphibian cells. As in mammals, lysine 16 is acetylated first, followed by 8 and/or 12 and then 5.

With lampbrush chromosomes from immature (previtellogenic) oocytes, antisera to H4 acetylated at lysines 8, 12, and 16 labeled fluorescent foci at the bases of transcription loops. Antisera to H4 acetylated at lysine 5 labeled weakly (i.e., the tri- and tetra-acylated isoforms must be rare). Loops showed weak labeling of the chromatin axis but intense fluorescence at particular points, which probably represent incompletely decondensed chromatin. The RNP matrix of loops, including the RNP-rich sphere bodies and the dense matrix of “marker” loops, was not labeled.

Treatment of immature oocytes with butyrate for 12 h to inhibit histone deacetylation did not affect immunolabeling, suggesting that turnover of H4 acetates is slow. In contrast, in chromosomes from mature oocytes, in which loops have retracted and transcription is low, butyrate caused an increase in labeling with all antisera, followed by the appearance of vestigial loops, weakly labeled, but with regions of intense fluorescence. These loops contain RNP and are presumably transcriptionally active. We conclude that H4 acetates turn over more rapidly in mature than immature oocytes and that histone hyperacetylation precedes, and possibly induces, loop formation and transcriptional activation.

Work carried out over many years in various experimental systems has shown that core histones extracted from transcriptionally active nuclei or associated with actively transcribed genes are often more highly acetylated than those from bulk chromatin (for review see Turner, 1991). However, there are exceptions to this general finding. For example, immunolabeling of insect polytene chromosomes with antisera to acetylated H4 has shown that very rapidly transcribing (puffed) chromatin is not labeled particularly strongly, although adjacent chromatin may be (Turner et al., 1990). Hyperacetylation of H4 cannot therefore be an integral and necessary part of the transcription mechanism in all eukaryotes. This conclusion is consistent with genetic experiments in the yeast Saccharomyces cerevisiae, where mutants carrying deletions of the amino-terminal domain of histone H4, which includes the acetylatable lysine residues, show normal expression of most of their genes (Kayne et al., 1988). Deletions of the amino-terminal domains of H2A, H2B, and H3 are also, individually, without major effects on transcription or viability (Wallis et al., 1983; Schuster et al., 1986; Durrin et al., 1991).

The posttranslational acetylation of the core histones is regulated by the balanced activities of chromatin-associated acetylating and deacetylating enzymes (Matthews and Waterborg, 1985; Attisano and Lewis, 1990). It is frequent (30% or more of H4 is acetylated in some cell types), ubiquitous among eukaryotes and energy requiring, all of which suggest that it plays an important role in the life of eukaryotic cells. However, it is not yet clear what this role might be, nor is it understood how changes in the acetylation of core histones may influence chromatin structure and function. Two general mechanisms, not mutually exclusive, can be envisaged. The first is that acetylation results directly in structural changes in chromatin. Early studies on the physical properties of isolated nucleosome core particles showed only small acetylation-related changes (for review see Turner, 1991). More recently, comparison of the ability of hyperacetylated and control histones to assemble circular plasmid DNA into nucleosomes has revealed differences consistent with some unwinding of core DNA from acetylated histone octamers (Norton et al., 1989; 1990). It remains to be seen what, if any, are the functional consequences of this change (Roberge et al., 1991) and indeed whether it occurs at all in vivo. The second possibility is that histone acetylation serves to alter the nucleosome surface such that binding of nonhistone proteins (NHPs)1 is either facilitated or suppressed.

1. Abbreviations used in this paper: AUT, acid-urea-Triton; NHP, nonhistone protein.
with consequent effects on chromatin function. A clear precedent for a biologically significant interaction between histone and NHPs has been established by experiments with H4 mutants in S. cerevisiae. These have demonstrated that silencing of mating type genes (particularly HMLα) requires binding of the SIR3 protein to the amino-terminal domain of H4 and that lysine 16, one of four acetylable residues in this domain, plays a particularly important role in mediating the effects of this binding (Kayne et al., 1988; Johnson et al., 1990; Megee et al., 1990; Park and Szostak, 1990; Johnson et al., 1992). Direct evidence is lacking for in vivo histone-NHP binding in other species, but it may be significant that the Drosophila DNA-binding, heterochromatin-forming protein Suvar (3')7 contains stretches of acidic amino acids resembling those found in known histone-binding proteins, which could well be used to bind to nucleosomal histones as part of a chromatin condensation mechanism (Reuter et al., 1990).

To investigate in detail the functional effects of histone acetylation, antisera have been prepared which can distinguish H4 molecules acetylated at each one of the four acetylable lysine residues 5, 8, 12, and 16, a distinction which cannot be made by conventional protein separation methods (Turner et al., 1989; Turner and Fellows, 1989). These antisera have been used to examine the distribution of acetylated H4 in Drosophila polytene chromosomes by indirect immunofluorescence microscopy to establish whether H4 molecules acetylated at one or another of the four lysines are associated with specific, functionally defined regions of the genome (Turner et al., 1992). The results show quite clearly that they are. Thus, H4 molecules acetylated at lysines 5 or 8 are found in similar but nonidentical islands distributed throughout the genome, except in heterochromatin where they are rare. H4 acetylated at lysine 12 is present in heterochromatin and elsewhere in the genome, while H4 acetylated at lysine 16 is found, almost exclusively, in the transcriptionally hyperactive X chromosome in male larvae. These results emphasize that in attempting to understand how histone acetylation regulates chromatin function, particularly transcription, it is important to know which lysine residues are acetylated and in what chromosome location.

The lambrush chromosomes which can be isolated from amphibian oocytes provide clearly separated regions of chromatin which are transcriptionally active (loops) or inactive (chromomeres) and have been widely used for the analysis of transcription by microsomal techniques (Sommerville, 1981; Callan, 1986). They are particularly suitable for studying the distribution of proteins within transcriptionally active chromatin by immunomicroscopy. In the present report, we use immunofluorescence microscopy to define the distribution of acetylated H4 in lambrush chromosomes and investigate the structural and functional consequences of histone hyperacetylation in cells treated with the deacetylase inhibitor sodium butyrate.

Materials and Methods

Antisera

Antibodies specific for acetylated forms of histone H4 were produced in rabbits using synthetic peptides corresponding to regions of the NH2 terminus of H4 acetylated at different lysine residues (Turner and Fellows, 1989; Turner et al., 1989). Specificities and the extents of the H4 epitopes recognized by the different antisera used in this study have been described in detail elsewhere (Turner et al., 1989; 1992). Specificities of rabbit antisera to the RNP components p22 and pp56 have also been described (Sommerville, 1981; Cummings et al., 1989). In brief, anti-p22 was directed against a 22-kDa protein component of heterogeneous nuclear RNP isolated from oocytes of Triturus cristatus carnifex (Sommerville, 1981) and anti-pp56 directed against a 56-kDa phosphoprotein component of poly(A)" RNP isolated from oocytes of Xenopus laevis (Cummings et al., 1989).

Cultured Cells

XTC-2 cells were derived from a metamorphosing tadpole of X. laevis (Pudney et al., 1973) and 7CC-1 cells were derived from abdominal skin cells of T. cristatus carnifex (Rudak, 1976). Both cell lines were cultured at 25°C in GMEM medium (Gibco-BRL Gaithersburg, MD) and 10% FCS. Hyper-acetylation of histones was induced by growing the cells for 12 h in medium containing 4 mM sodium butyrate. For immunostaining, cells were grown on the surface of sterilized glass coverslips for 24-48 h before use.

Oocytes

Ovary was dissected from female T. cristatus and maintained in modified Barth's solution as described previously (Johnson et al., 1984). Individual oocytes were manually defolliculated and sorted into groups of early vitellogenic (white, 0.4-0.5 mm) and full-grown (pale green, 1.5 mm). Hyper-acetylation of histones was induced by maintaining the oocytes at 20°C for 4 or 20 h in the presence of 40 mM sodium butyrate (Partington et al., 1984).

Immunostaining

Slides carrying blood smears or coverslips carrying cultured cells were immersed in methanol for 10 min at -20°C, and then in acetone for 5 min at -20°C. After several washes in TBS followed by TBS plus 5% calf serum, the cells or chromosomes were covered with antiserum diluted between 1:50 and 1:1,000 with TBS/1% calf serum, and a coverslip was placed on top. The preparations were incubated at 20°C for 60 min in a humid chamber, and then rinsed four times (each for 5 min), in TBS, FITC-conjugated anti-rabbit IgG (Amersham International, Amersham, UK) diluted 1:200 in TBS/1% calf serum was then applied to each preparation which was incubated at 20°C for 30 min. After rinsing again four times in TBS, the preparations were mounted in 50% glycerol containing 1 mg/ml phenylenediamine to prevent bleaching. Cells and chromosomes were examined with a Leitz Ortholux II fluorescence microscope and photographed on Kodak T-max 3200 film, using oil immersion or water immersion optics for fluorescence.

Histone Extraction and Analysis

Cells were pelleted from culture medium by centrifugation at 300 g for 5 min. The cells were rinsed in PBS (137 mM NaCl, 27 mM KCl, 1 mM MgCl2, 5 mM Na2HPO4, 1.5 mM KH2PO4), pelleted, and raised again in PBS adjusted to 0.5% Triton X-100 and 0.1% PMSF. The cell suspension was placed on ice for 10 min with occasional agitation and was checked for complete lysis by microscopy. The nuclei were pelleted by centrifugation at 600 g for 5 min and extracted with 10 vol of 0.22 M HCl overnight at 4°C. After centrifugation, the soluble extract was recovered and 0.25 ml added to 750 ml. Aliquots of the crude histone extracts were run on polyacrylamide gels containing acetic acid, urea, and Triton X-100 (AUT gels) as described previously (Bonner et al., 1980; Turner and Fellows, 1989). Histone H4 from calf thymus (Boehringer Mannheim Corp., Indianapolis, IN) or histones from human HL-60 cells (Turner et al., 1989) were used as electrophoretic standards. Proteins were electrophoretically transferred from gels to nitrocellulose filters either in 0.7% acetic acid after...
cells. However, to interpret the results of such experiments, it has been possible to use these antisera to locate H4, and identifies H4 molecules acetylated at lysine 5 and 12 has been described in detail elsewhere (Turner et al., 1989; Munks et al., 1991). Each one of the antisera used in this study recognizes a single site of acetylation in histone H4 and their specificities have been described in detail elsewhere (Turner et al., 1989; Munks et al., 1991). It is possible to calculate the frequency of acetylation at each lysine residue without reference to protein content, provided antibody binding (a) values are obtained with all four site-specific antisera. For each of the four antisera, binding to each isoform is expressed as a percentage of the value for the tetraacetylated isoform (a4) which is set to 100% (all four lysines being fully acetylated in this isoform). This procedure normalizes all binding values and eliminates the inevitable differences between antisera due to antibody titre or dilution. Because immunostaining is more sensitive than antibody binding, a value for a4 is calculated by multiplying the measured binding of R12/8 to the triacetylated isoform (a3) by the ratio of the tetraacetylated and the triacetylated isoforms. Because lysine 16 is fully acetylated in the tetraacetylated isoform (see below), the ratio a14/a16 provides an accurate measure of this ratio.

**Results**

Each one of the antisera used in this study recognizes a single site of acetylation in histone H4 and their specificities have been described in detail elsewhere (Turner et al., 1989; 1992). The number assigned to each antiserum defines both the animal from which the serum was obtained and the specificity. Thus, antiserum R6/5 was obtained from rabbit 6 and identifies H4 molecules acetylated at lysine 5 (H4Ac5). Because of the extreme evolutionary conservation of H4, it has been possible to use these antisera to locate acetylated H4 in both mammalian and insect chromosomes by immunoblotting and the experiments described below show that the same approach can be applied to amphibian cells. However, to interpret the results of such experiments, it is necessary to confirm the specificity of each antiserum in the species under study and to establish the order in which individual lysine residues are acetylated.

**Patterns of H4 Acetylation in Amphibian Cells**

Histones were extracted from *Xenopus* XTC-2 cells and separated by electrophoresis in AUT gels, a technique which clearly resolves both the different histones and the four acetylated isoforms of H4. Typical results are shown in Fig. 1. In untreated cells (lane 2) H4 consisted primarily of the nonacetylated and monoa cetylated isoforms (69 and 27%, respectively, calculated from laser scans of lanes stained with Coomassie blue). Overnight treatment with sodium butyrate, an inhibitor of histone deacetylases, increased H4 acetylation so that the acetylated isoforms collectively accounted for 87% of cellular H4 (lane 1). This increase in acetylation is greater than that noted previously in *Xenopus* X58 cells after comparable butyrate treatment (Candido et al., 1978), indicating a more rapid turnover of H4 acetates in XTC-2 cells.

To test the specificity of our antisera for acetylated H4 in amphibian cells and to define the frequency with which different H4 lysines are acetylated in these cells, histones were electrophoresed from AUT gels onto nitrocellulose filters and immunostained with antisera to H4 acetylated at each of the four acetylatable lysines (i.e., residues 5, 8, 12, and 16). Results are presented in Fig. 1. All antisera, including R5/12 (not shown in Fig. 1 but used for several immunostaining experiments), recognized *Xenopus* H4. R20/12 detected no other proteins (lanes 8 and 9). R14/16 (lanes 10 and 11) occasionally revealed faint bands migrating faster than the nonacetylated isoform, possibly H4 breakdown products. Similar bands have been seen in extracts from both mammalian and insect cells (Turner et al., 1992). Two slowly migrating bands were detected by R6/5 and R12/8. With R12/8 (lanes 6 and 7), these each accounted for only a small percentage of total labeling (4-7% in untreated cells and 20% after butyrate treatment). R6/5 (lanes 4 and 5) labeled H4 from untreated cells very weakly and, because of this, the slowly migrating bands accounted for about half the total labeling. However, in extracts from butyrate-treated cells...
that the order in which different lysines are acetylated is not influenced to any great extent by butyrate treatment. The values for butyrate-treated and untreated cells show that the order in which different lysines are acetylated at one of the four sites (as indicated in the top left corner) for each isoform.

![Figure 2. Frequency of acetylation at lysines 5, 8, 12, and 16 in the mono-, di-, tri-, and tetraacetylated isoforms of H4 from Xenopus XTC-2 cells. Each one of the four vertical bars shows the frequency of acetylation at one of the four sites (as indicated in the top left corner) for each isoform.](image)

We conclude from these findings that antisera R6/5, R12/8, R5/12, R20/12, and R14/16 all recognize the acetylated isoforms of H4 from amphibian cells and show little or no cross-reaction with other proteins.

Butyrate treatment increased labeling of the acetylated isoforms of H4 by up to fourfold. By scanning autoradiographs derived from butyrate-treated extracts, it is possible to quantify antibody binding to each of the acetylated isoforms and to calculate the level of acetylation of each lysine residue in each isoform, as described in Materials and Methods. From this, the order in which sites are acetylated can be deduced. Results are summarized in Fig. 2. The monoacetylated isoform is acetylated predominantly at lysine 16. The diacetylated isoform is acetylated at lysines 16, 12, or 8 (the apparent drop in acetylation at Lys 16 in the diacetylated isoform is probably not significant) while acetylation of Lys 5 remains low. In the triacetylated isoform lysines 8, 12, and 16 are acetylated to about the same extent and about twice as frequently as Lys 5. This pattern of site usage is remarkably similar to that found in cultured cells from humans and other mammals (Turner et al., 1989; Thorne et al., 1990) but differs from the patterns seen in cuttlefish testis, Tetrahymena micro- and macro-nuclei, and Drosophila (Couppez et al., 1987; Chicoine et al., 1986; Munks et al., 1991).

The method for calculating the frequency with which different lysines are acetylated requires measurement of labeling of the tetraacetylated isoform, which is present in sufficient amounts only in butyrate-treated cells. It is therefore possible that the pattern of site usage has been distorted by butyrate treatment. To test this, we compared the labeling, with each antiserum, of the monoacetylated isoform from butyrate-treated and untreated cells. If butyrate does indeed change the order in which lysines are acetylated, then this should be detected as differences in antibody labeling per unit of protein (the protein present in each isoform being measured by scanning lanes stained with Coomassie blue). Values (arbitrary units) for butyrate-treated and untreated cells (respectively) were as follows: R6/5, 4.5:7; R12/8, 3.5:5.0; R20/12, 2.0:3.5; R14/16, 13.3:14.4. The similarity of the values for butyrate-treated and untreated cells shows that the order in which different lysines are acetylated is not influenced to any great extent by butyrate treatment.

**Immunostaining of Cells Active and Inactive in Transcription**

Antiserum R5/12 was used in initial studies on immunostaining reactions with amphibian cells. A comparison was made between actively growing cultured cells and nongrowing, terminally differentiated erythrocytes. Nuclei of *Xenopus* XTC-2 cells were found to be highly reactive with R5/12 at a dilution of 1:50 (Fig. 3, a and b). In contrast, nuclei of *Xenopus* erythrocytes react weakly with R5/12 at this dilution (Fig. 3, c and d).

The conclusion that this difference in immunostaining reflects differences in the level of H4 acetylation is strengthened by results with an antiserum raised against a nonacetylated H4 NH2-terminal peptide, (R15/0, Turner et al., 1989). This antiserum, at a dilution of 1:50, labels erythrocyte nuclei (Fig. 3, e and f) at least as strongly as XTC-2 cell nuclei (not shown). Thus, the weak labeling of erythrocytes with R5/12 is not due simply to inaccessibility of H4 in the chromatin of these cells. Since XTC-2 cells are active in transcription, whereas erythrocytes are largely transcriptionally inert, the difference in immunostaining of acetylated H4 may relate to the transcriptional state of the chromatin.

**Use of H4 Acetylation Sites in Active and in Butyrate-treated Cells**

Antibodies recognizing different sites of H4 acetylation were used to immunostain amphibian cultured cells. Relative fluorescence intensities with antisera R12/8, R5/12 (or R20/12), R13/16, and R6/5 indicate that H4 lysines 8, 12, and especially 16 are more frequently acetylated in *Xenopus* cultured cells than is Lys 5 (Fig. 4, a, c, and e). These findings are entirely consistent with the Western blotting results presented in Figs. 1 and 2, which show that lysine 16 is the most frequently acetylated residue in *Xenopus* H4 while lysine 5 is rarely used. Essentially, the same results were obtained on using the much larger cultured cells of *Triturus* (Fig. 4, g, i and k), indicating that H4 acetylation patterns are the same in these widely diverged amphibians.

The extent of hyperacetylation after treatment of cultured cells with sodium butyrate (4 mM for 12 h) was also determined for each antiserum. A dramatic increase in acetylation at Lys 12 was seen with both R5/12 (not shown) and R20/12 (Fig. 4, d and f), at Lys 16 with R13/16 (Fig. 4, f and i), and at Lys 8 with R20/12 (not shown). In contrast, the overall level of immunostaining with R6/5 is minimally changed by butyrate treatment, though increased immunostaining of structure within nucleoli is noticeable (Fig. 4 b). As expected, immunostaining with the antiserum raised against a nonacetylated H4 peptide (R15/0) is not increased after butyrate treatment (cf. Fig. 4, g and h).

**Immunostaining of Regions of Transcriptionally Active and Inactive Chromatin in Lampbrush Chromosomes**

To determine possible relationships between H4 acetylation and specific regions of transcriptional activity within chromatin, use was made of lampbrush chromosomes from oocytes of the newt *Triturus*. In the chromosomes isolated from early vitellogenic oocytes of this animal, a clear distinction can be made between regions of transcriptionally active chromatin (lateral loops) and regions of condensed chromatin (chromomeres). Each lateral loop consists of an extended...
Figure 3. Immunofluorescent staining of Xenopus XTC-2 cells and erythrocytes using antisera R5/12 and R15/0. (a and b) Phase-contrast and fluorescence images of XTC-2 cells immunostained with R5/12. (c and d) Phase-contrast and fluorescence images of erythrocytes immunostained with R5/12. (e and f) Phase-contrast and fluorescence images of erythrocytes immunostained with R15/0. All antiserum dilutions are 1:50. Bar, 20 μm.

chromatin axis surrounded by a matrix of RNP transcripts, whereas the chromomeres contain up to 95% of the chromatin packaged into dense structures deployed along the chromosome axes (for review see Callan, 1986).

The different site-specific antisera were used to immunostain lambrush chromosomes. An unexpected result is that R12/8 (Fig. 6), R5/12, and R20/12 and R13/16 (not shown) all immunostain chromomeres, i.e., condensed chromatin. There is little distinction between staining with these different antisera and the following description for R12/8 also applies for R5/12 and R13/16.

On close examination, it is seen that the chromomeres are not immunostained evenly with R12/8: distinct fluorescent foci can be seen within the masses of chromomeric chromatin (Fig. 6, b and d). These foci generally are located at the points of insertion of lateral loops into the chromomeres. Occasionally, the fluorescent foci are seen as pairs lying across the width of the chromomeres (Fig. 6, d and g) each pair presumably representing highly acetylated sites on the two chromatids at the bases of homologous loop pairs.

The much finer structures of the loop axes, representing chromatin fibers actually being transcribed, immunostain with R12/8 much less intensely than do chromomeric structures (Fig. 6, b and d). However, nodes of more intense fluorescence are seen at points around the axis of many loops (Fig. 6 e). In general, the fluorescent nodes do not correspond to any obvious feature of loop morphology. Although it is possible that some of these nodes are special sites of incompletely decondensed chromatin since essentially the same pattern is seen with antibodies directed against DNA (Scheer, 1987). An extreme example of incompletely decondensed loop chromatin, with an immunostained lump occurring about half-way around each component of the loop pair, is seen in Fig. 6, a and b. Various loop structures with atypical distributions of RNP matrix have been mapped to specific chromosomal loci (Callan and Lloyd, 1960). “Dense-matrix” loops (Fig. 6, c and d) and “sphere” loci (Fig. 6, f and g), both of which carry masses of RNP material, do not fluoresce with antibodies directed against acetylated H4. Although the matrices of the vast majority of lambrush loops consist of nascent transcripts, the spherical structures seen at the sphere organizers and also occurring free in the nucleoplasm consist mainly of RNA splicing factors (Gall and Callan, 1989). Therefore, the location of acetylated H4 is site specific with regard to both chromomeric and loop substructures.

In contrast to the immunostaining with R12/8, R5/12, R20/12, and R13/16, and as observed with cultured cells, R6/5 immunostains lambrush chromosomes poorly, the only distinct fluorescence being seen at sites within extra-chromosomal nucleoli (Fig. 5, c and d). From this we can conclude that the tri- and tetraacetylated isoforms of H4 (the only ones which R6/5 recognizes) are rare in lambrush chromosomes, even in the chromomeres. R15/0 also gave weak immunostaining, the most noticeable reaction occurring with the matrix of the lateral loops, although a weak chromomeric reaction can be discerned (Fig. 5, e and f). It should be noted that R15/0 has a lower titre than the antisera Sommerville et al. H4 Acetylation and Transcription 281
Figure 4. Immunofluorescent staining of Xenopus XTC-2 cells and Triturus TCC-1 cells. Xenopus cells (a-f) and Triturus cells (g-l) were immunostained with antisera R6/5 (a and b), R20/12 (c, d, i and j), R13/16 (e, f, k and l), and R15/0 (g and h). Cells which had been treated with sodium butyrate (4 mM for 12 h) are shown (b, d, f, h, j and l) beneath untreated cells (a, c, e, g, i and k). All antiserum dilutions are 1:200. Bar, 20 μm.

to acetylated H4 and stains comparatively weakly at a dilution (× 200) at which background staining is acceptably low.

To confirm that the loop reaction seen with antisera to acetylated H4 is indeed recognizing the chromatin axis of the loops, a comparison was made between these antisera and antisera specific for the RNP matrix of the loops. As shown in Fig. 7, R5/12 (b) reacts with a much finer axial component of large loops than does anti-p22 (c), which reacts with the
thicker loop matrix and also with extrachromosomal RNP particles resulting from shed loop matrix.

Treatment of early vitellogenic oocytes with sodium butyrate (40 mM for 4–6 h) had little effect on either the structure of the chromosomes or the extent of acetylation, as judged by immunostaining with R12/8 (Fig. 6, h and i), R13/16 (not shown), and R20/12 (not shown). R6/5 remained poorly reactive with lampbrush chromosomes after butyrate treatment (not shown). In addition to little apparent increase in acetylation, butyrate treatment produced no obvious increase in parameters that relate to transcriptional activity, such as loop length, matrix density, or rate of incorporation of \(^{3}H\)uridine into RNA (not shown). Collectively, these results lead to the conclusion that the acetylation/deacetylation cycle in lampbrush chromosomes of early vitellogenic oocytes is operating slowly.

**Reactivation of Condensed Chromatin**

In the chromosomes of full-grown oocytes, loops are no longer present, the chromomeres are compact, and chromomeric fusion brings about a shortening of the chromosomes. These features signal cessation of transcription and progressive condensation of chromatin in preparation for meiotic segregation of homologous chromosomes. Immunostaining of condensed chromosomes with antisera R20/12 (Fig. 8, a and b), R12/8, and R13/16 (not shown) reveals that the chromomeres still contain acetylated H4, although, as judged by the relative intensity of fluorescence, acetylation levels are lower than seen in the chromomeres of transcriptionally active chromosomes. A less subjective indication of lower levels of acetylation is given by comparison of the fluorescence intensities obtained with antisera to nonacetylated and
acetylated H4 (compare b and f in Fig. 5 and b and c in Fig. 8).

Treatment of full-grown oocytes with sodium butyrate (40 mM for 4–12 h) brings about dramatic changes in chromosome structure. The chromomeres become swollen, reaching diameters of about three times those seen in chromosomes from untreated oocytes. This swelling is seen to best advantage on immunostaining the chromosomes with R13/16 (Fig. 8, f and g), R20/12 (Fig. 8, i and j), and R12/8 (not shown) which also reveals much increased levels of H4 acetylation. In fact, the chromomeres react with antisera to acetylated H4 at least as strongly as those in lampbrush chromosomes at their maximum involvement in transcription (cf. Fig. 5 b).

That hyperacetylation precedes chromatin swelling can be seen in chromosomes isolated from full-grown oocytes after 4 h of butyrate treatment. In such preparations the chromosomes are still condensed with chromomeric fusion resulting in shortened chromosomes (Fig. 8 d). However, immunostaining with R13/16 already gives very intense fluorescence (Fig. 8 f). Furthermore, antiserum R6/5 now immunostains the chromomeres with a reaction more intense (Fig. 8 e) than seen in lampbrush chromosomes (cf. Fig. 5 d). As lysine 5 is acetylated only in the most highly acetylated isoforms (H4Ac3 and H4Ac4) it can be concluded that the condensed chromosomes of full-grown oocytes are much more predisposed to hyperacetylation on addition of sodium butyrate than are lampbrush chromosomes. This suggests an increased rate of turnover of H4 acetates, either as a result of accumulation of histone acetylases and deacetylases during oogenesis, and/or changes in the accessibility of potential acetylation sites as the chromatin is prepared for meiotic segregation.

In addition to the swelling and increased immunoreactivity of the chromomeres, the chromatin from butyrate-stimulated oocytes appears to unravel at the fringes of the chromomeres, (Fig. 8 g) and eventually (after 12 h of treatment) forms de novo, small lateral loops. These vestigial loops are difficult to see by phase contrast (Fig. 8 h) but are clearly revealed by immunostaining (Fig. 8, i and j). As in lampbrush chromosomes, it appears to be a thin, axial component of the loops, presumably the chromatin fiber, that immunostains. Also, as seen with lampbrush chromosomes, small immunostained nodes are located along the loop axes which in this instance may represent chromatin in the process of unraveling (Fig. 8, i and j).

That the vestigial loops are engaged in transcription is indicated by their immunostaining with antisera raised against an mRNA-bound phosphoprotein (pp56, Cummings et al., 1989). This antisera binds specifically to the loop matrix of lampbrush chromosomes (Fig. 9, a and b). After the cessation of transcription in full-grown oocytes, im-

---

**Figure 6.** Immunofluorescent staining of lampbrush chromosomes with R12/8. (a and b) Phase and fluorescence images of two chromosomal regions showing reactive foci within chromomeres (ch) and reactive dense structures about halfway around a pair of unusual loops (arrowheads). (c and d) Phase and fluorescence images of a chromosomal region containing nonreactive denser matrix loops (black arrowheads). Paired fluorescent chromomeric foci are also seen (white arrowheads). (e) Fluorescence image of loops showing reactive nodes around the loop axes (arrowheads). (f and g) Phase and fluorescence images of chromosomal regions containing ‘sphere organizers,’ showing nonreactive sphere structures (sp). Paired fluorescent chromomeric foci are again indicated (white arrowheads). (h and i) Phase and fluorescence images of chromosomes derived from oocytes treated with 40 mM sodium butyrate for 4 h. Antiserum dilution is 1:1,000. Bar, 10 μm.
Figure 8. Immunofluorescent staining of chromosomes isolated from full-grown oocytes. The oocytes were either untreated (a–c), or treated with 40 mM sodium butyrate (d–j). (a) Phase-contrast image of chromosomes lacking lateral loops. no, nucleolus. (b) Immunostaining of a with R20/12. (c) Immunostaining of a preparation similar to a with R15/0. (d) A more compacted chromosome bivalent derived from an oocyte treated with sodium butyrate for 4 h. (e) Immunostaining of chromosomes in d with R6/5. Note that a similar pattern of brightly fluorescent bands is present in each of the bivalents (examples are arrowed). (f) Bivalents treated with sodium butyrate as in e but immunostained with R13/16. (g) Part of a bivalent isolated from an oocyte treated for 12 h with sodium butyrate and immunostained with R13/16.
progresses from the nonacetylated to the tetraacetylated iso-
ocytes which are treated with butyrate (our unpublished
and Sommerville, 1988), it seems likely that the vestigial
detected in association with mRNA sequences (Cummings
anti-pp56 (Fig. 9, e and f). Since the protein pp56 is usually
results). To demonstrate conclusively that de novo loop for-
munoreaction no longer occurs with the chromosomes (Fig.
show an increase in the rate of RNA synthesis in full-grown
omomere spools out at least one
H4 acetylated at lysine 16, which detect all acetylated iso-
forms (25–30% of total H4), would be expected to label
strongly and to be less sensitive to butyrate treatment than
the other antisera. These predictions are entirely consistent
with the immunolabeling results presented here.

Immunolabeling of Triturus cultured cells with the same
panel of antisera to acetylated H4 gave results very similar
to those obtained with Xenopus cells, confirming that the or-
der in which individual lysines are acetylated is the same in
H4 from these two widely diverged amphibians. Morphologi-
cally superior lampbrush chromosomes are obtained from
Triturus oocytes and we chose to use these to pinpoint sites
of H4 acetylation within chromatin substructures.

The Chromomere As a Sensor for Chromatin
Modification and Loop Formation

Previous studies on immunostaining lampbrush chromo-
somes with antibodies raised against native histone mole-
cules have shown strong reactions with the chromomeres
(Scheer et al., 1979). This is only to be expected, for at least
95% of the chromatin is contained in these structures. How-
ever, in the earlier experiments (as in the one shown here in
Fig. 5 f) antibodies raised against nonacetylated histones
reacted, to variable extents, with the RNP matrix of the
loops. It has been noted previously that antibodies to
nonacetylated H4 (antiserum R15/0) label the transcription-
ally active chromatin of polytene chromosome puffs particu-
larly strongly (Turner et al., 1990; and our unpublished
results), a result which has been interpreted as reflecting the
greater accessibility of the H4 amino-terminal region in
extended, transcriptionally active chromatin. While improved
accessibility may also contribute to labeling of the lamp-
brush chromosome loops, it is likely that attachment of
stored histones to the RNP matrix during chromosome iso-
lation is also a major factor (as discussed by Sommerville,
1981). Histones are stored in large amounts in growing oo-
cytes and it has been reported that most stored H4 is di-
acetylated (Woodland, 1979). However, we have been unable
to detect labeling of stored H4 on Western blots with antisera
to acetylated H4. Only antiserum R15/0, which detects
nonacetylated H4, gave significant labeling (our unpublished
results). Although the reasons for this discrepancy remain
unclear, the results indicate that nonspecific association of
stored H4 with lampbrush chromosome loops would en-
force immunolabeling with R15/0, but not with antisera to
acetylated H4.

The most surprising aspect of immunostaining lampbrush
chromosomes is the extent of reaction of some of the site-
specific antibodies with the chromosomes. Usually the chro-
nomeres are considered to be "silent" domains of inactive
chromatin (for review see Callan, 1986), and so the results
presented here do not tally exactly with the commonly held
view that H4 acetylation is selective for regions of transcrip-
tion. Nevertheless, each chromomere spools out at least one

Note the swelling and disaggregation of chromomeres which remain connected by thin interchromomeric fibers, and the unraveling
of chromatin at the fringes of chromomeres (arrowheads). (h) Part of a bivalent after treatment with butyrate for 12 h. (i) Immunostaining
of chromosomes in h with R20/12. (j) A similar preparation to that in h and i from an oocyte treated in the same way, also immunostained
with R20/12 and showing small lateral loops immunostained both on thin lateral fibrils (arrowheads) and on periodic blobs of incompletely
extended chromatin. All antiserum dilutions are 1:500. Bar, 10 μm.
Figure 9. Immunofluorescent staining of lateral loops with anti-pp56. (a and b) Immunostaining of lampbrush chromosomes from early vitellogenic oocytes showing specificity for loop RNP matrix. (c and d) Immunostaining of chromosomes from full-grown oocytes showing absence of RNP. (e and f) Immunostaining of chromosomes from full-grown oocytes which have been treated with 40 mM sodium butyrate for 12 h showing reappearance of RNP matrix on vestigial loops (arrows). Antiserum dilution is 1:500. Bar, 10 μm.

A pair of lateral loops and although not active in transcription itself, does provide the anchorage points of the loops. It is these very anchorage points which appear to be the sites most heavily acetylated. This structural relationship could be extended to a functional one if the base of the loop is considered to act as a sensor, or target, for components required for loop formation and maintenance. Acetylation of H4 in the chromomeres and in selected regions of the loop chromatin may be required to keep the chromatin in a (potentially) relaxed or accessible state in which transcription can be induced as required. The regions of chromatin actually transcribed (i.e., the transcription units in loops) would be determined by other, transcription-specific factors. In this respect, it is interesting to note that in *Drosophila*, H4 isoforms acetylated at particular lysine residues serve to mark functionally discrete chromatin domains (Turner et al., 1992). Accumulating circumstantial evidence suggests that these acetylated isoforms may act through binding to specific NHPs, a role for which the exposed amino-terminal domain of H4 is ideally suited (discussed by Turner, 1991; Turner et al., 1992). Whatever may be the role of H4 acetylation in transcriptionally active lampbrush chromosomes, it clearly does not require rapid turnover of acetate groups. The finding that the deacetylase inhibitor sodium butyrate did not induce H4 hyperacetylation in early vitellogenic oocytes shows that most H4 acetates must turn over slowly at this stage of development.

De Novo Generation of Loops

The interpretation that general changes in histone acetylation within and around transcription units precede more specific determinations of transcription is consistent with the observations that chromosomes lacking loops can be hyper-acetylated by treating living oocytes with sodium butyrate and that these chromosomes eventually generate transcriptionally active loops. In these experiments, three stages can be discerned: (1) increased acetylation of H4 at all sites, including lysine 5, a site used only in the more highly acetylated isoforms, (2) swelling of the chromomeres, and (3) generation of vestigial loops from the fringes of the relaxed chromomeres (see Fig. 8). Further evidence—namely, increased rates of RNA synthesis and the presence of RNP proteins on the new loops—indicates that loop transcription is being reinitiated, although autoradiographic analysis would be required to prove this point.
The observations made in this report show that massive hyperacetylation of chromatin-bound H4 can occur in full-grown oocytes. Since the raison d'être of the oocyte is to accumulate the diverse materials required to make an embryo, it is likely that the full-grown oocyte has accumulated nuclear acetylases and deacetylases to power chromatin transformations occurring during the remainder of meiosis, after fertilization and through the many early embryonic cell divisions.

**Comparison of H4 Acetylation in Lampbrush and Polytene Chromosomes**

It is interesting to compare the immunolabeling results described here with those reported recently using polynucleosomes from Chironomid insects (Turner et al., 1990; 1992). Although polynucleosomes represent quite a different biological situation—being derived from interphase somatic cells as opposed to diploplote meiotic cells—they do share the very useful feature of exhibiting regions of high transcriptional activity (described as puffs and Balbiani rings) which can be studied by light microscopy.

In interpreting the immunolabeling patterns of polynucleate and lampbrush chromosomes, it is important to bear in mind that the order in which individual lysines are acetylated in these two chromosome types is very different. Whereas in amphibian H4, lysines are acetylated in a set order (16 then 12 or 8 then 5), in *Drosophila* the monoaacetylated isoform can be acetylated at any one of these four residues (Munks et al., 1991). Immunolabeling experiments suggest that this is attributable to the existence of site-specific acetylases distributed in separate locations throughout the *Drosophila* genome, rather than to the activity of a ubiquitous, nonsite-specific H4 acetylase (Turner et al., 1992). Thus, the banded immunolabeling pattern given by antiserum R6/5 on *Drosophila* polynucleate chromosomes shows the distribution, primarily, of H4 molecules in which a single acetate group is attached to lysine 5. This, in turn, is likely to reflect the distribution of a lysine 5–specific acetylase. In contrast, the banded immunolabeling pattern given by the same antiserum on chromosomes from mature oocytes treated with sodium butyrate (Fig. 8 e) shows the location of tri- and tetraacetylated H4, as these are the only isoforms in which lysine 5 is acetylated in significant amounts in amphibian cells. Chromatin domains enriched in tri- and tetraacetylated H4 after butyrate treatment must be those in which rapid turnover of H4 acetates is occurring.

In both lampbrush and polynucleate chromosomes, regions of chromatin undergoing transcription are not uniformly enriched in acetylated H4. Puffs, Balbiani rings, and lampbrush loops are all weakly labeled with antisera to acetylated H4, relative to their labeling with antisera to the nonacetylated isoform. However, regions adjacent to transcribing chromatin do contain acetylated H4. These include puff boundaries and in lampbrush chromosomes, the points of loop insertion in chromomeres and small domains within the loops themselves. In both chromosome types it seems that H4 acetylation is associated with the initiation and maintenance of the decondensed chromatin state which is first established before the onset of transcription, rather than in the process of transcription itself. To what extent acetylated H4 exerts its functional effects through directly influencing chromatin structure or through selective binding of NHPs remains to be established.

We are grateful to Jayne Lavender for carrying out the Western blotting experiments. This work was supported by grants from the U.K. Cancer Research Campaign and the Wellcome Trust.

Received for publication 25 June 1992 and in revised form 28 September 1992.

**References**

Antissono, L., and P. N. Lewis. 1990. Purification and characterization of two porcine liver nuclear histone acetyltransferases. *J. Biol. Chem.* 265:3949–3955.

Barrett, P., R. M. Johnson, and J. Sommerville. 1984. Immunological identity of proteins that bind stored 5sRNA in Xenopus oocytes. *Exp. Cell Res.* 153:299–307.

Bonner, W. M., M. H. P. West, and D. Stedman. 1980. Two-dimensional gel analysis of histones in acid extracts of nuclei, cells, and tissues. *Eur. J. Biochem.* 109:17–23.

Callan, H. G. 1986. Lampbrush Chromosomes. Springer-Verlag, Berlin, Germany. 254 pp.

Callan, H. G., and L. Lloyd. 1960. Lampbrush chromosomes of crested newts. *Triturus cristatus* (Laurenti). *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 245:135–219.

Candido, E. P. M., R. Reeves, and J. R. Davie. 1978. Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell.* 14:105–113.

Chao, M. L., G. O. Schulman, R. Richman, R. G. Cook, and C. D. Allis. 1986. Nonrandom utilization of acetylation sites in histones isolated from Tetrahymena. Evidence for functionally distinct H4 acetylation sites. *J. Biol. Chem.* 261:1071–1076.

Couper, M., A. Martin-Poussiehou, and P. Sautdier. 1987. Histone H4 from cuttlefish testis is sequentially acetylated. Comparison with acetylation of calf thymus histone H4. *J. Biol. Chem.* 262:2854–2860.

Cummings, A., and J. Sommerville. 1988. Protein kinase activity associated with stored messenger ribonucleoprotein particles of Xenopus oocytes. *J. Biol. Chem.* 107:45–46.

Cummings, A., P. Barrett, and J. Sommerville. 1989. Multiple modifications in the phosphoproteins bound to stored messenger RNA in Xenopus oocytes. *Biochim. Biophys. Acta.* 1014:319–326.

Durrin, L. K., R. K. Mann, P. S. Kayne, and M. Grunstein. 1991. Yeast histone H4 N-terminal sequence is required for promoter activation in vivo. *Cell.* 65:1023–1031.

Gall, J. G., and H. G. Callan. 1989. The sphere organelle contains small nuclear ribonucleoproteins. *Proc. Natl. Acad. Sci. USA.* 86:6635–6639.

Johnson, R. M., P. Barrett, and J. Sommerville. 1984. Distribution and utilization of SS-RNA-binding proteins during the development of Xenopus oocytes. *Eur. J. Biochem.* 144:503–508.

Johnson, L. M., P. S. Kayne, E. S. Kahn, and M. Grunstein. 1990. Genetic evidence for an interaction between SR3 and histone H4 in the repression of the silent mating loci in *Saccaromyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 87:6286–6290.

Johnson, L. M., G. Fisher-Adams, and M. Grunstein. 1991. Identification of a nonbasic domain in the histone H4 N-terminus required for promoter activation in vivo. *Cell.* 65:1023–1031.

Matthews, H. R., and J. H. Waterborz. 1985. Reversible modifications of nuclear proteins and their significance. In *The Enzymology of Post-translational Modification of Proteins.* Vol. 2. Academic Press Inc., London. 125–185.

Megee, P. C., B. A. Morgan, B. A. M. Mitanooa, and M. M. Smith. 1990. Genetic analysis of histone H4: essential role of lysines subject to reversible acetylation. *Science (Wash. DC).* 247:841–845.

Munks, R. L. J., J. Moore, L. P. O'Neill, and B. M. Turner. 1991. Histone H4 acetylation in *Drosophila*. Frequency of acetylation at different sites defined by immunolabeling with site-specific antibodies. *FEBS (Feb. Eur. Biochem. Soc.) Lett.* 284:245–248.

Norton, V. G., B. S. Imai, P. Yau, and E. M. Bradbury. 1989. Histone acetylation reduces nucleosome core particle linking number change. *Cell.* 57:449–457.

Norton, V. G., K. W. Marvin, P. Yau, and E. M. Bradbury. 1990. Nucleosome linking number change controlled by acetylation of histone H3 and H4. *J. Biol. Chem.* 265:19468–19473.

Park, E. C., and J. W. Szostak. 1990. Point mutations in the yeast historic H4 gene prevent silencing of the silent mating locus HML. *Mol. Cell. Biol.* 10:4932–4934.
Partington, G. A., N. J. Yarwood, and T. R. Rutherford. 1984. Human globin gene transcription in injected Xenopus oocytes: enhancement by sodium butyrate. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2787-2792.

Pudney, M., M. G. R. Varma, and C. J. Leake. 1973. Establishment of a cell line (XTC-2) from the South African clawed toad, *Xenopus laevis*. *Experientia* (Basel). 29:466-467.

Reuter, G., M. Giarre, J. Farah, J. Gausz, A. Spierer, and P. Spierer. 1990. Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc-finger protein. *Nature (Lond.)*. 344:219-223.

Roberge, M., T. E. O'Neill, and E. M. Bradbury. 1991. Inhibition of 5S RNA transcription *in vitro* by nucleosome cores with high or low levels of histone acetylation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 288:215-218.

Rudak, E. 1976. The structural organization of newt mitotic chromosomes. Ph.D. thesis, University of St. Andrews, Scotland. 8-25.

Scheer, U. 1987. Structure of lampbrush chromosome loops during different states of transcriptional activity as visualized in the presence of physiological salt concentrations. *Biol. Cell.* 59:33-42.

Scheer, U., J. Sommerville, and M. Bustin. 1979. Injected histone antibodies interfere with transcription of lampbrush chromosome loops in oocyte of *Pleurodeles*.* J. Cell Sci.* 40:1-20.

Schuster, T., M. Han, and M. Grunstein. 1986. Yeast histone H2A and H2B amino termini have interchangeable functions. *Cell.* 45:445-451.

Sommerville, J. 1981. Immunolocalization and structural organization of nascent RNP. In The Cell Nucleus. Vol. 8: H. Busch, editor. Academic Press Inc., New York. 1-57.

Thorne, A. W., D. Knicek, K. Mitchelson, P. Sauti, and C. Crane-Robinson. 1990. Patterns of histone acetylation. *Eur. J. Biochem.* 191: 701-713.

Turner, B. M. 1991. Histone acetylation and control of gene expression. *J. Cell Sci.* 99:13-20.

Turner, B. M., and G. Fellows. 1989. Specific antibodies reveal ordered and cell cycle-related use of histone H4 acetylation sites in mammalian cells. *Eur. J. Biochem.* 179:131-139.

Turner, B. M., L. P. O'Neill, and I. M. Allan. 1989. Histone H4 acetylation in human cells. Frequency of acetylation at different sites defined by immunolabeling with site-specific antibodies. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 253:141-145.

Turner, B. M., L. Franchi, and H. Wallace. 1990. Islands of acetylated histone H4 in polytene chromosomes and their relationship to chromatin packaging and transcriptional activity. *J. Cell Sci.* 96:335-346.

Wallis, J. W., M. Rykowski, and M. Gronstein. 1983. Yeast histone H2B containing large amino terminus deletions can function in vivo. *Cell.* 35: 711-719.

Woodland, H. R. 1979. The modification of stored histones H3 and H4 during the oogenesis and early development of *Xenopus laevis*. *Dev. Biol.* 68:360-370.