Histone H4 Acetylation and Transcription in Amphibian Chromatin

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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-acetylated 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.

W ORK 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,

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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 acetylatable 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 acetylatable 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 lampbrush 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 microscopical 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 lampbrush 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-kD protein component of heterogeneous nuclear RNP isolated from oocytes of *Triturus cristatus carnifex* (Sommerville, 1981) and anti-pp56 directed against a 56-kD 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 TCC-1 cells were derived from abdominal skin cells of *T.c. carnifex* (Rudak, 1976). Both cell lines were cultured at 25°C in GMEM medium (Gibco-BRL Gaithersburg, MD) and 10% FCS. Hyperacetylation 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.c. carnifex* 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). Hyperacetylation 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).

Lampbrush Chromosomes

Nuclei were released from punctured oocytes into 3:1 solution (75 mM KCl, 25 mM NaCl, 10 mM Tris-HCl, pH 7.4). The nuclei were transferred to bored disc wells containing 3:1 solution supplemented with 0.1 mM CaCl2 and chromosomes were released, allowed to spread, and centrifuged as described previously (Scheer et al., 1979). Chromosome preparations were immersed in 70% ethanol for 5 min, and then rinsed in TBS (120 mM NaCl, 10 mM Tris-HCl, and pH 7.4) before immunostaining.

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 mM 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 urea was added to 750 mg/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

equilibration of gels in this solution for 2 h, or as previously described (Turner and Fellows, 1989). Immunoblotting was carried out using primary antibodies at a dilution of 1:200 in TBS, 5% BSA and either ^{125}I-labeled protein A (0.2 µCi/ml, 30 mCi/mg; Amersham International) as described previously (Barrett et al., 1984) or ^{125}I-labeled goat anti-rabbit IgG (Amersham International) at 10[6] cpm/ml.

Calculation of Frequency of Acetylation at Individual H4 Lysines

We have previously described how one can calculate the frequency with which lysines 5, 8, 12, and 16 are acetylated in each of the mono-, di-, and triacetylated isoforms of H4 (Turner et al., 1989; Munks et al., 1991). Briefly, the procedure involves Western blotting from AUT gels, immunostaining with site-specific antisera to acetylated H4 and ^{125}I-labeled antibodies to rabbit IgG, and scanning of autoradiographs to quantify the binding of each site-specific antiserum to each acetylated isoform. (This is defined as a_xn, where x is the lysine residue and n is the isoform). These values are then converted to antibody binding per unit of protein, the amount of protein in each acetylated isoform being calculated from scans of parallel lanes stained with Coomassie blue. Values of antibody binding per unit of protein can be used to determine the frequency with which each lysine is acetylated in each isoform by setting the value for the tetraacetylated isoform to 100% for each antiserum and expressing values for the other isoforms as a proportion of this. This procedure is dependent on the accurate determination of the amount of protein in the tetraacetylated isoform, which can be difficult with cell types in which butyrate treatment fails to generate large amounts of tetraacetylated H4, or which contain proteins with similar electrophoretic mobilities to this isoform.

It is possible to calculate the frequency of acetylation at each lysine residue without reference to protein content, provided antibody binding (a_xn) 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 (a_x4) 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 Coomassie blue, reliable estimates of a_x4 can be made, even when the tetraacetylated isoform is present at low levels. The values calculated in this way provide an estimate of the relative frequencies with which different lysine residues are acetylated in each isoform. To convert these to absolute values for the percentage acetylation at each lysine we can use the fact that these values must add up to 100, 200, 300, and 400 for the mono-, di-, tri-, and tetraacetylated isoforms, respectively. Although this procedure effectively eliminates problems due to estimating the protein content of each isoform, a difficulty remains with antiserum R12/8, where binding to the tetraacetylated isoform (a_84) is consistently lower than expected, due to inhibition by acetylation of the adjacent lysines, 5 and/or 12 (Turner et al., 1989; see Fig. 1). To allow for this, a value for a_84 is calculated by multiplying the measured binding of R12/8 to the triacetylated isoform (a_83) by the ratio of the tetraacetylated and the triacetylated isoforms. Because lysine 16 is fully acetylated in the triacetylated isoform (see below), the ratio $a_{16}4:a_{16}3$ 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 immunomicroscopy 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
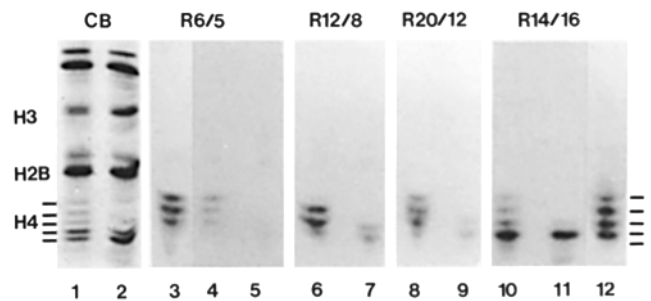

Figure 1. Detection by Western blotting of acetylated isoforms of H4 extracted from *Xenopus* XTC-2 and human HL60 cells and separated by electrophoresis on AUT gels. XTC-2 extracts were run in pairs, in each of which the sample on the left is from butyrate-treated cells (lanes *1, 4, 6, 8,* and *10*) and that on the right from untreated cells (lanes *2, 5, 7, 9,* and *11*). The remaining two lanes (*3* and *12*) contain extracts from butyrate-treated human HL60 cells run in parallel. The lanes shown were stained with Coomassie blue (*1* and *2*) or transferred to nitrocellulose filters and immunostained with antisera R6/5 (*3–5*), R12/8 (*6* and *7*), R20/12 (*8* and *9*), and R14/16 (*10–12*).

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 monoacetylated 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 ∼70% 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 electroblotted 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 >2% 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
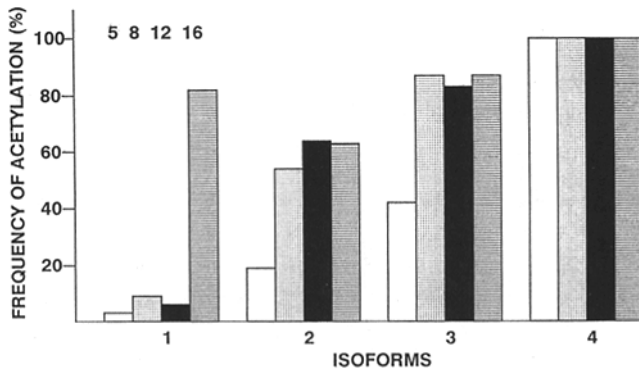
Figure 2. Frequency of acetylation at lysines 5, 8, 12, and 16 in the mono-, di-, tri-, and tetraacetylated isoforms of H4 from *Xenopus* XTC-2 cells. Each one of the four vertical bars shows the frequency of acetylation at one of the four sites (as indicated in the top left corner) for each isoform.

acetylated H4 accounted for ∼90% of labeling with R6/5. 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 two 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, 4.5:7.0; 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 *j*), at Lys 16 with R13/16 (Fig. 4, *f* and *l*), and at Lys 8 with R12/8 (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 ooctyes 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
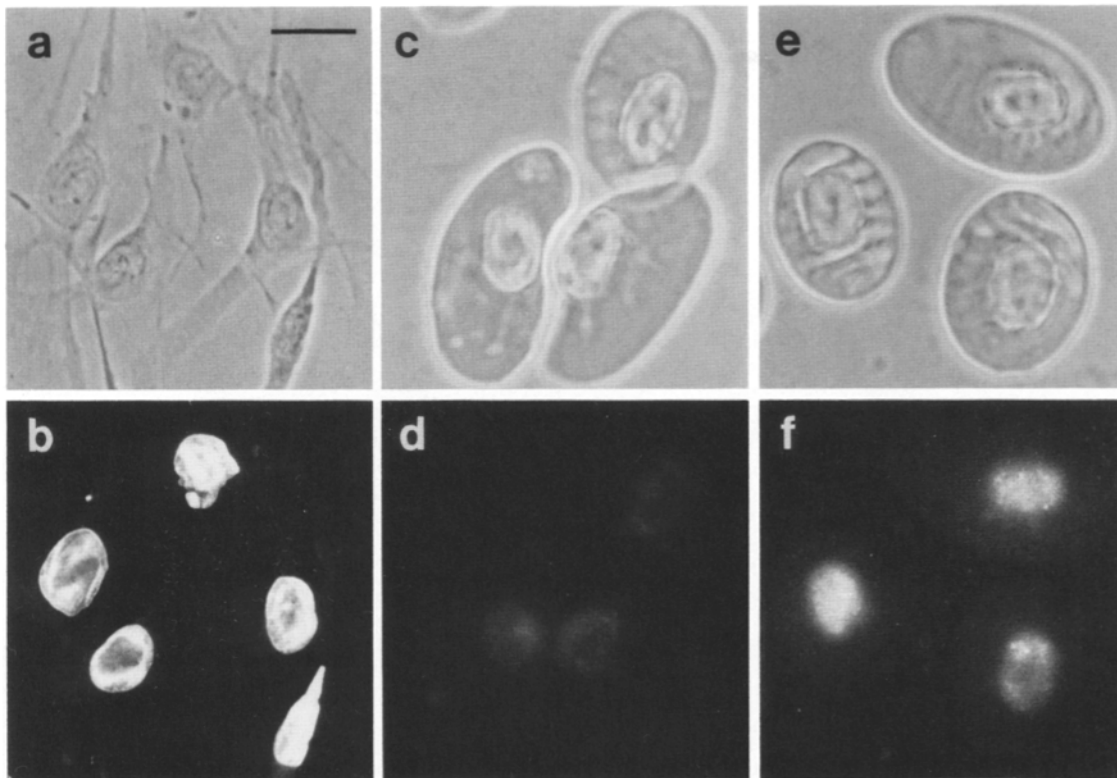
Figure 3. Immunofluorescent staining of *Xenopus* XTC-2 cells and erythrocytes using antisera R5/12 and R15/0. (*a* and *b*) Phase-contrast and fluorescence images of XTC-2 cells immunostained with R5/12. (*c* and *d*) Phase-contrast and fluorescence images of erythrocytes immunostained with R5/12. (*e* 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 lampbrush chromosomes. An unexpected result is that R12/8 (Fig. 6), R5/12, (Fig. 5, *a* and *b* and Fig. 7, *a* and *b*), 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 hyperacetylated H4, it is more likely that they represent

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 lampbrush 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 lampbrush chromosomes poorly, the only distinct fluorescence being seen at sites within extrachromosomal 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 lampbrush 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
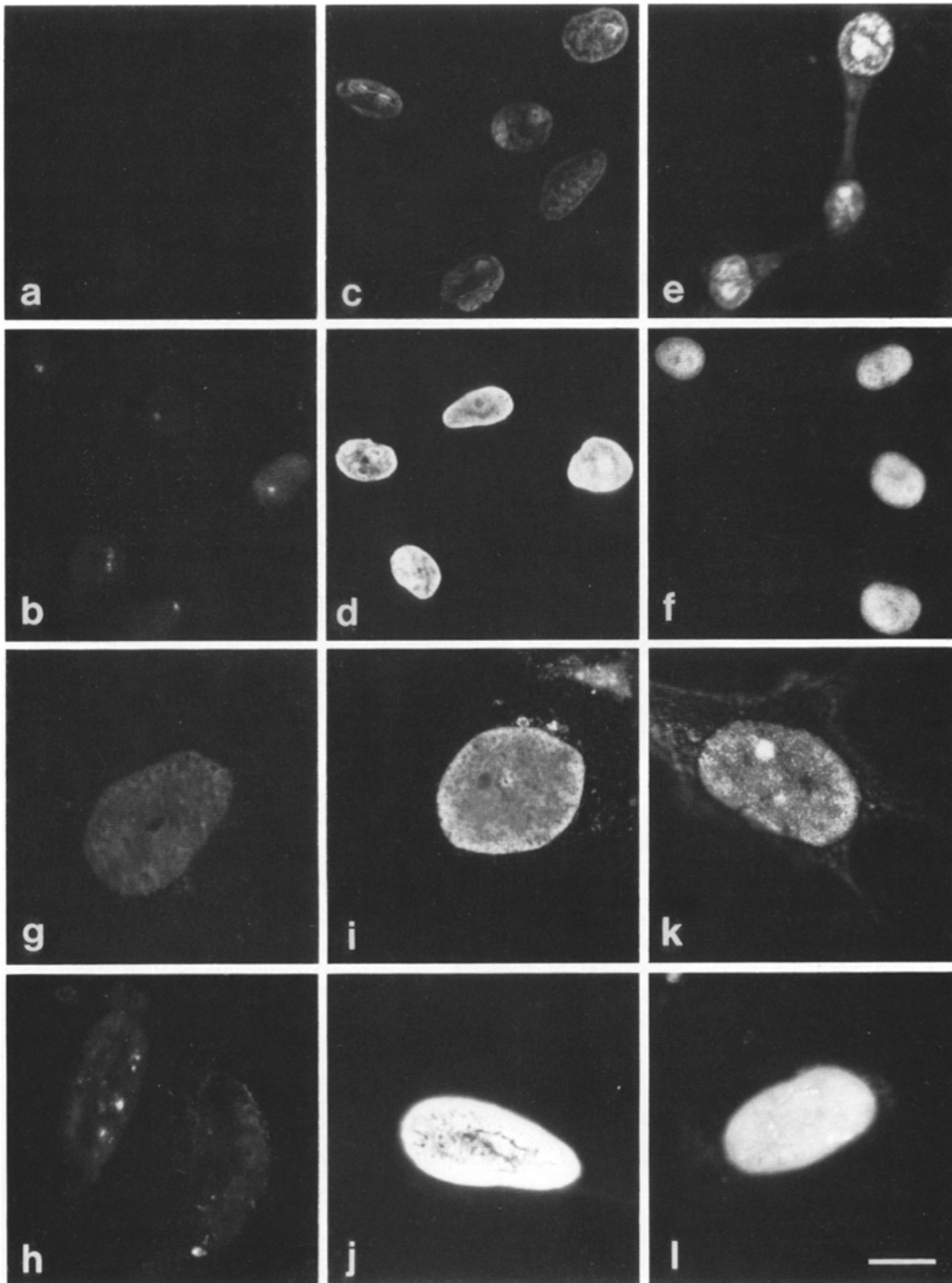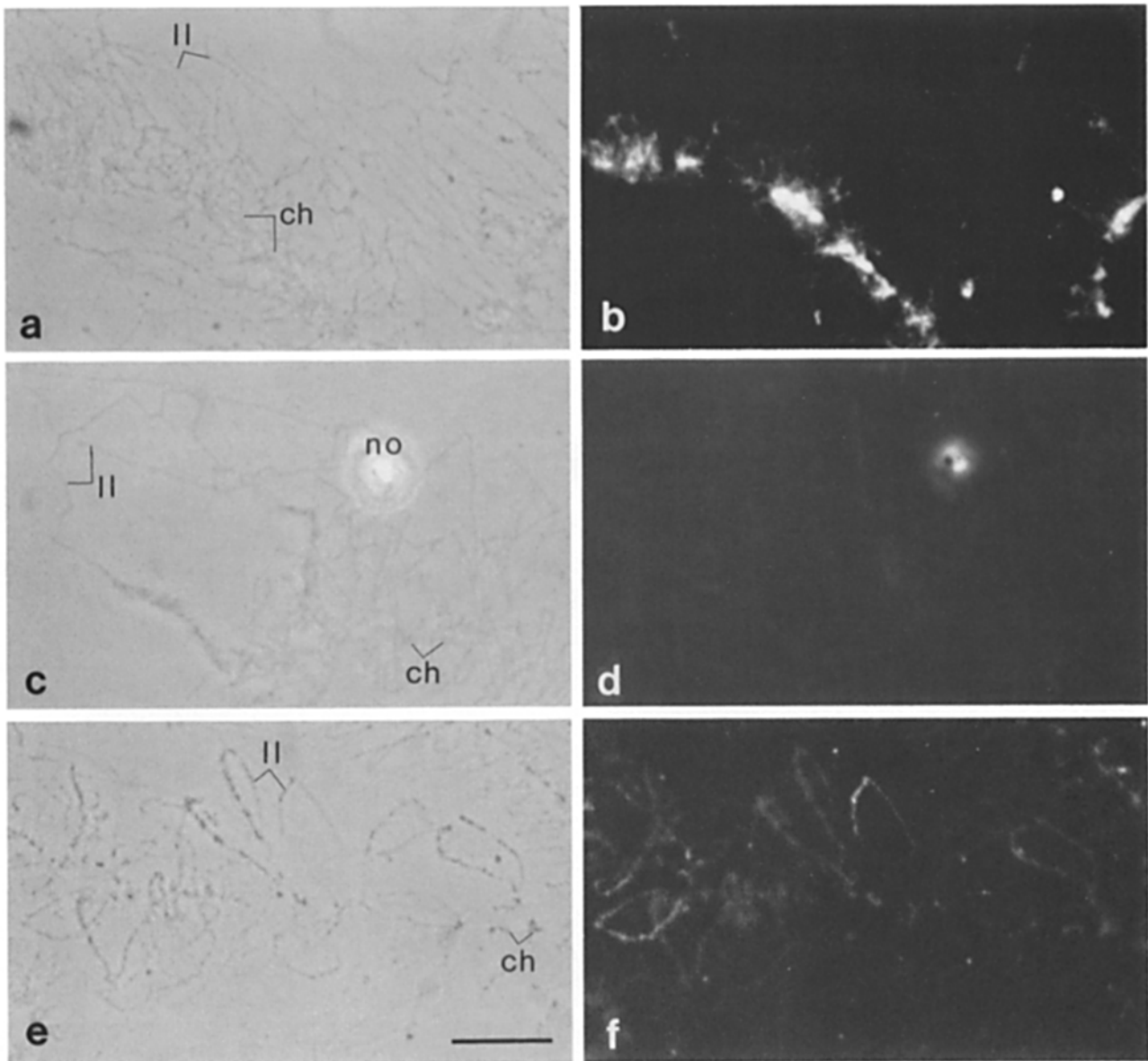
Figure 4. Immunofluorescent staining of *Xenopus* XTC-2 cells and *Triturus* TCC-1 cells. *Xenopus* cells (*a–f*) and *Triturus* cells (*g–l*) were immunostained with antisera R6/5 (*a* and *b*), R20/12 (*c, d, i* and *j*), R13/16 (*e, f, k* and *l*), and R15/0 (*g* and *h*). Cells which had been treated 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
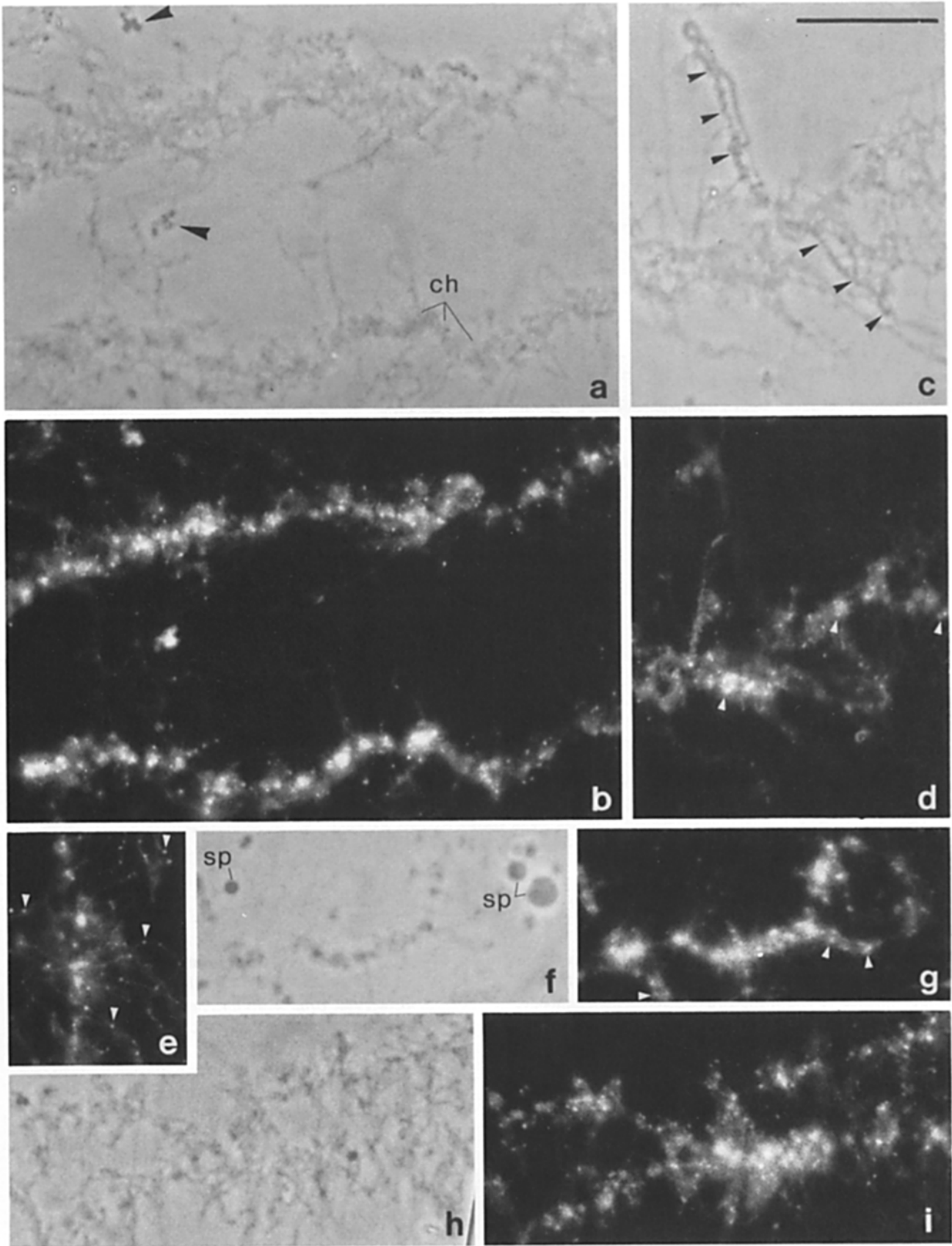
Figure 5. Immunofluorescent staining of lampbrush chromosomes. (*a* and *b*) Phase and fluorescence images of chromosomes immunostained with R5/12. (*c* and *d*) Phase and fluorescence images of chromosomes immunostained with R6/5. (*e* and *f*) Phase and fluorescence images of chromosomes immunostained with R15/0. All antiserum dilutions are 1:500 *ll*, lateral loops; *ch*, chromomeres; *no*, nucleolus. Bar, 10 μm.

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 [³H]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

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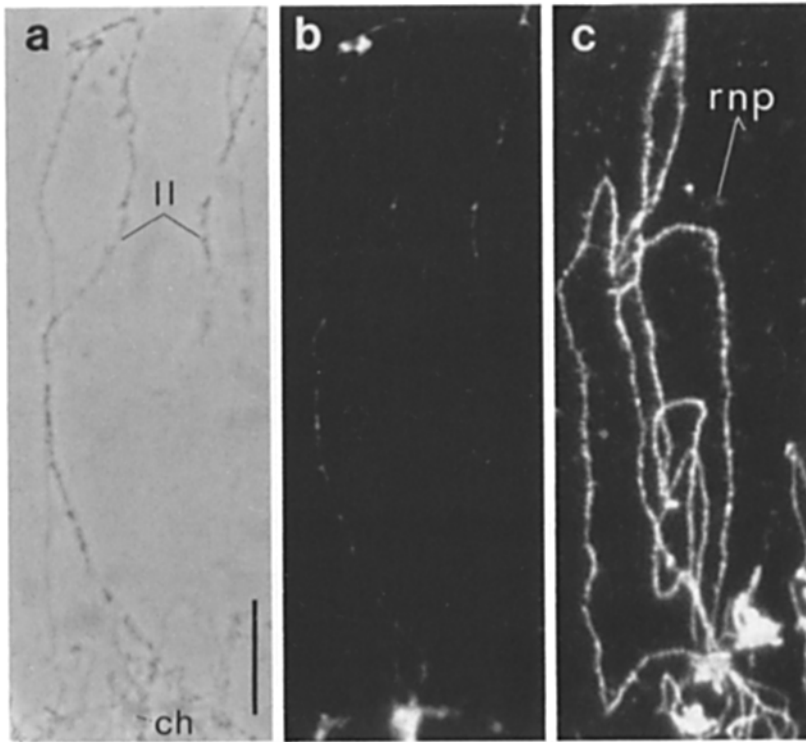
Figure 7. Immunofluorescent staining of lamp-brush loops. (*a*) Phase-contrast image of a pair of large lateral loops. (*b*) Immunostaining of loops and chromomere in *a* with R5/12. (*c*) Immunostaining of loops similar to those in *a* with anti-p22. Antiserum dilutions are 1:500. Abbreviations: *ll*, lateral loops; *ch*, chromomere; *rnp*, free ribonucleoprotein aggregates. Bar, 10 μm.

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 predis-

posed 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 antiserum raised against an mRNA-bound phosphoprotein (pp56, Cummings et al., 1989). This antiserum 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.
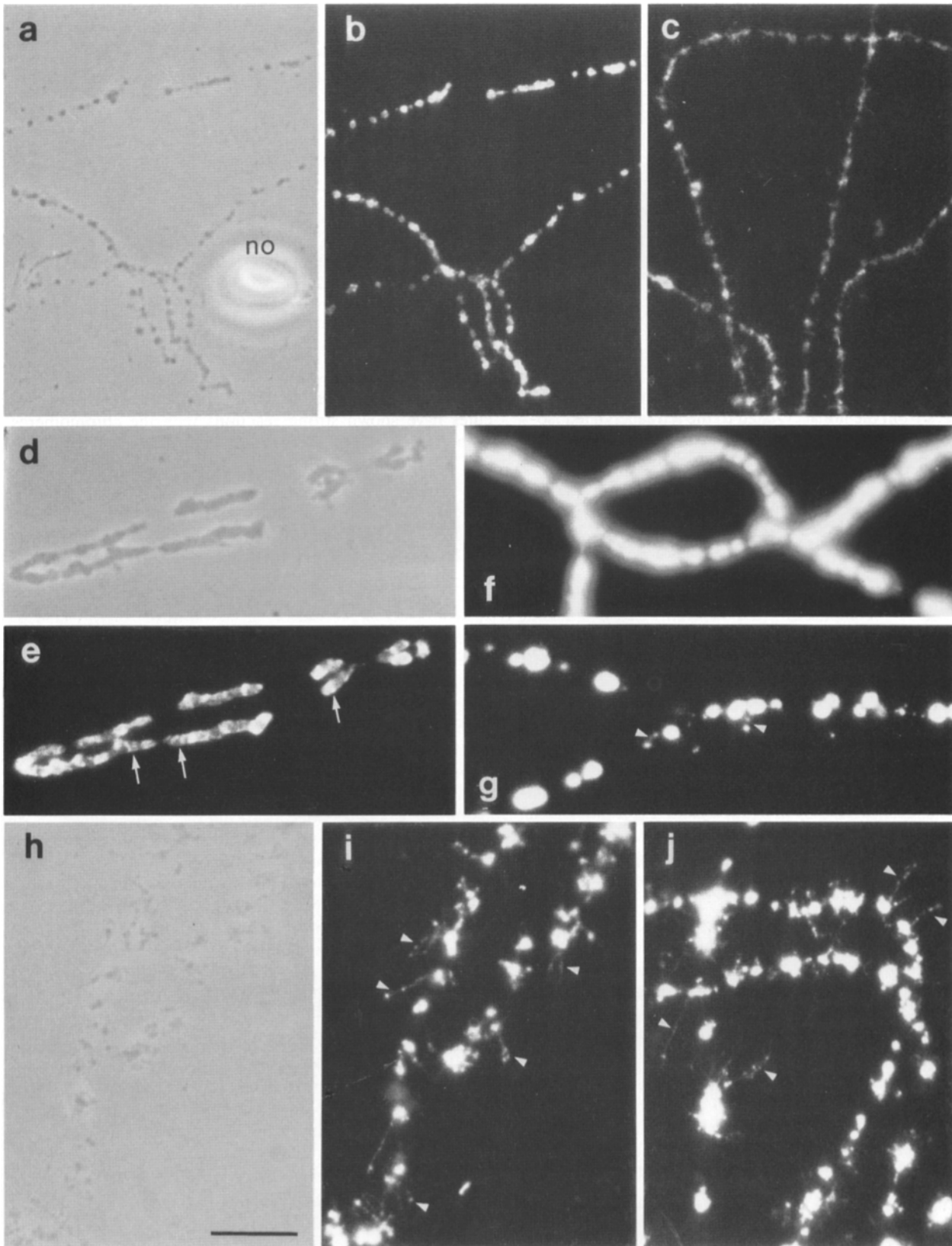
Figure 8. Immunofluorescent staining of chromosomes isolated from full-grown oocytes. The oocytes were either untreated (*a–c*), or treated with 40 mM sodium butyrate (*d–j*). (*a*) Phase-contrast image of chromosomes lacking lateral loops. *no*, nucleolus. (*b*) Immunostaining of *a* with R20/12. (*c*) 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.

munoreaction no longer occurs with the chromosomes (Fig. 9, c and d). However, after treatment of full-grown oocytes with sodium butyrate (40 mM for 12 h), the small lateral loops which have been stimulated to form now react with anti-pp56 (Fig. 9, e and f). Since the protein pp56 is usually detected in association with mRNA sequences (Cummings and Sommerville, 1988), it seems likely that the vestigial loops bear a matrix of RNP transcripts. Preliminary results show an increase in the rate of RNA synthesis in full-grown oocytes which are treated with butyrate (our unpublished results). To demonstrate conclusively that de novo loop formation involves transcription will require autoradiographic studies.

Discussion

Work with several species has revealed differences in the order in which individual lysines are acetylated as H4 progresses from the nonacetylated to the tetraacetylated isoform (Chicoine et al., 1986; Couppez et al., 1987; Turner et al., 1989, Thorne et al., 1990; Munks et al., 1991). This variation between species in patterns of H4 acetylation cannot be due simply to differences in the accessibility of individual lysines within the nucleosome, the structure of which changes little from one species to another, but is more likely to reflect differences in the specificities of the acetylating enzymes.

We show here that the order in which H4 lysines are acetylated in Xenopus is remarkably similar to that in human cells. Thus, lysine 16 is the most frequently acetylated residue in monoacetylated H4; lysine 16 and either 8 or 12 can be used in diacetylated H4; while lysine 5 is little used until the tri- and tetraacetylated isoforms. In light of these results, we can conclude that immunolabeling with antisera to H4 acetylated at lysine 16 (H4Ac16) reflects the distribution of all acetylated isoforms of H4, of which the monoacetylated is the most common. Antisera to H4Ac8 and H4Ac12 (i.e., R12/8, R5/12, and R20/12) locate the di-, tri-, and tetraacetylated isoforms while chromatin which is immunolabeled with antibodies to H4Ac5 (antiserum R6/5) is enriched in tri- and tetraacetylated H4 (i.e., is hyperacetylated).

In view of the rarity of tri- and tetraacetylated H4 in untreated cells (Fig. 1, lane 2), it is not surprising that R6/5 immunolabels Xenopus culture cells, generally, very weakly. Even after treatment with butyrate to increase histone acetylation, the tri- and tetraacetylated isoforms are relatively uncommon and staining should only be intense in regions where turnover of acetate groups is particularly rapid. It seems that chromatin within or adjacent to the nucleolus falls into this category (Figs. 4 b and 5 d). Antisera to H4 acetylated at lysines 8 and/or 12 should label more intensely than R6/5 because of their ability to detect the more common diacetylated isoform, but should still be considerably enhanced by butyrate treatment. On the other hand, antisera to

H4 acetylated at lysine 16, which detect all acetylated isoforms (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 order in which individual lysines are acetylated is the same in H4 from these two widely diverged amphibia. Morphologically 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 chromosomes with antibodies raised against native histone molecules 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. However, 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 transcriptionally active chromatin of polytene chromosome puffs particularly 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 lampbrush chromosome loops, it is likely that attachment of stored histones to the RNP matrix during chromosome isolation is also a major factor (as discussed by Sommerville, 1981). Histones are stored in large amounts in growing oocytes and it has been reported that most stored H4 is diacetylated (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 enhance 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 chromomeres 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 transcription. Nevertheless, each chromomere spools out at least one

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Note the swelling and disaggregation of chromomeres which remain connected by thin interchromomeric fibrils, 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.
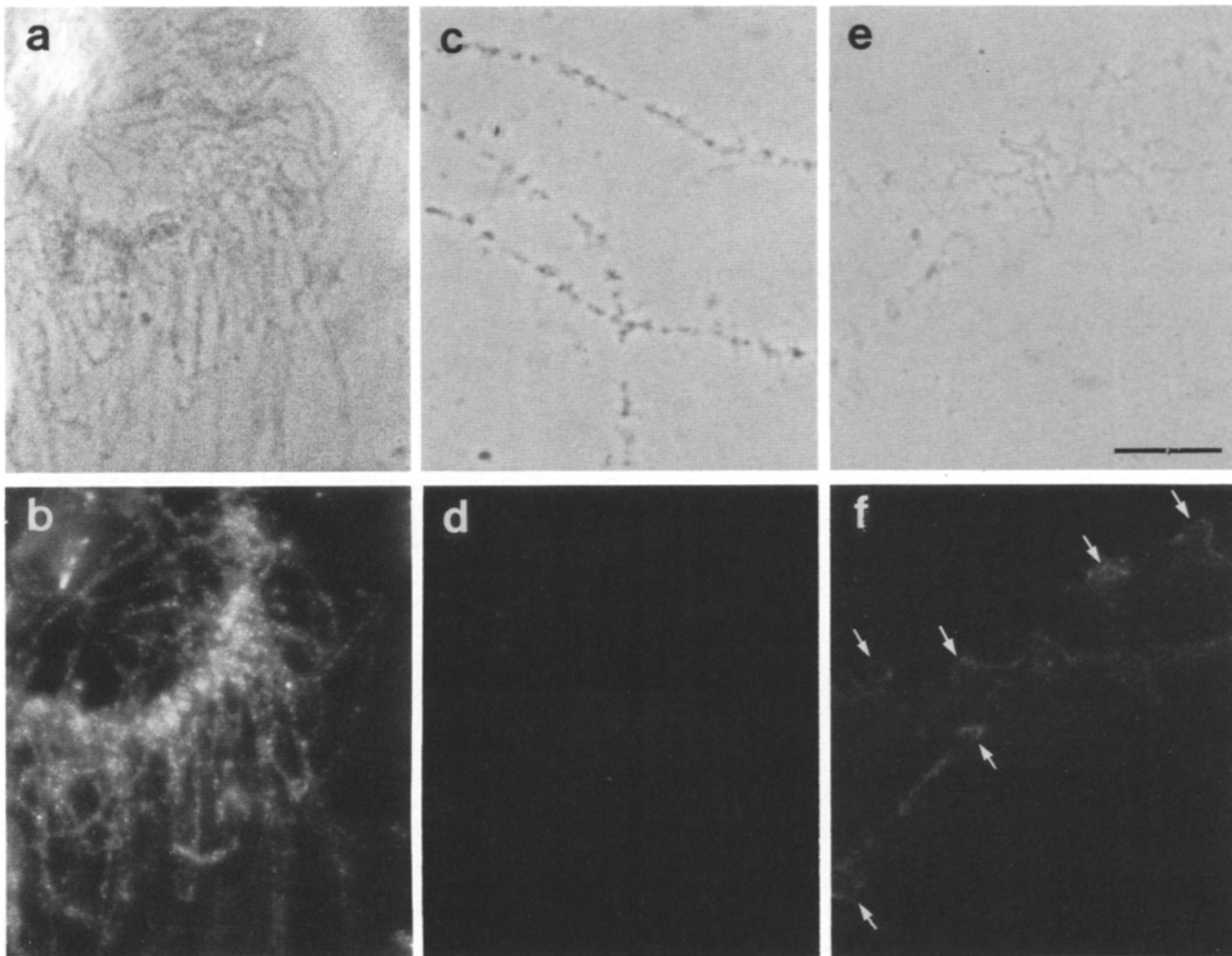
Figure 9. Immunofluorescent staining of lateral loops with anti-pp56. (*a* and *b*) Immunostaining of lampbrush chromosomes from early vitellogenic oocytes showing specificity for loop RNP matrix. (*c* and *d*) Immunostaining of chromosomes from full-grown oocytes showing absence of RNP. (*e* and *f*) 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.

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 hyperacetylated 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 polytene chromosomes from Chironimid insects (Turner et al., 1990; 1992). Although polytene chromosomes represent quite a different biological situation—being derived from interphase somatic cells as opposed to diplotene 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 polytene 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 amphibia H4, lysines are acetylated in a set order (16 then 12 or 8 then 5), in Drosophila the monoacetylated 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 polytene 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 tetra-acetylated 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 polytene 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 chro-

matin structure or through selective binding of NHPs remains to be established.

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