A homogeneous oligonucleosome complex was prepared by reconstitution of highly hyperacetylated histone octamers onto a linear DNA template consisting of 12 tandemly arranged 208-base pair fragments of the 5 S rRNA gene from the sea urchin Lytechinus variegatus. The ionic strength-dependent folding of this oligonucleosome assembly was monitored by sedimentation velocity and electron microscopy. Both types of analysis indicate that under ionic conditions resembling those found in the physiological range and in the absence of histone H1, the acetylated oligonucleosome complexes remain in an extended conformation in contrast to their nonacetylated counterparts. The implications of this finding in the context of a multistate model of chromatin folding (Hansen, J. C., and Ausio, J. (1992) TIBS 1797, 187-191) as well as its biological relevance are discussed.

In our opinion, one of the important landmarks in the chromatin field during the last two decades has been the realization that histones are not merely passive structural players but have an important functional role in the modulation of genomic expression (2-4). In other words, they have both a structural and a functional role.

At the structural level, histones provide the protein blocks that allow for the hierarchical folding of DNA in nucleosomes, higher order, and chromosomal structures. At the highest level of compaction the structures arising from such organization (chromosomes) may be essential to prevent the shearing of long eukaryotic DNA molecules during the transmission of the genetic material from one cell to another in the course of cell division (5, 6) in eukaryotes. At the lower levels, chromatin structure and nucleosomes may have also been selected during the evolutionary transition from prokaryotes to eukaryotes, as the scaffold that provides support and modulates the fine-tuning of the more complex functional mechanisms of transcription and replication. All these levels of folding must be reversible in order to accommodate the different functional needs during the cell cycle. The dynamic aspects of this chromatin folding and its implications both at the structural and functional level are still poorly understood.

During the cell cycle, histones undergo several chemical post-translational modifications that could presumably be involved in the modulation of chromatin folding. One of the more extensively characterized of such modifications has been histone acetylation. Histone acetylation is a dynamic post-translational metabolic modification (7) that has been strongly correlated with transcriptional activity and with the processes of histone deposition (such as during replication) or histone displacement (such as during spermatogenesis) (for detailed reviews see Refs. 8–11).

At the structural level, histone acetylation is a lysine amidation reaction catalyzed by acetyltransferases. By virtue of its chemical nature, it alters the net positive charge balance of the N-terminal regions (tails) of the core histones (histones H2A, H2B, H3, and H4), and as such, it has been long hypothesized to weaken the histone-DNA interactions involved at the different levels of chromatin folding. A disruption of the protein-DNA interactions would be expected to lead to the loss of stability (folding) of both the chromatin fiber and its constitutive subunits, the nucleosomes. Nevertheless, the experimental effort to provide support to this hypothesis has led in the past, quite often, to conflicting results. At its best it has provided evidence for only minor changes in chromatin folding.

The structural effect of histone acetylation on the conformation of the nucleosome core particle (12) (consisting of a double set of each H2A, H2B, H3, and H4 histones and 146-base pair DNA) has been found to be small (12-15). A larger effect was observed in the case of nucleosomes consisting of longer DNA (16). Very small charges were also reported at the higher order structure level of chromatin folding (17, 18).

Despite all this, there is increasing evidence that the major effect of the histone tails (and hence acetylation) on chromatin folding (19) arises from their interactions with the linker DNA region connecting adjacent nucleosomes in the chromatin fiber. We have recently proposed a coupled multistate model of chromatin dynamics to account for the contribution of chromatin folding in the modulation of genetic activity (1). The results that follow, while providing experimental support for this model, also point to the importance of acetylation in the mechanisms of chromatin folding and dynamics.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chicken blood was obtained from commercial slaughterhouses in Victoria and processed immediately after collection. The 208–12 DNA template consisting of 12 tandem repeats of a 208-base pair fragment of the 5 S rRNA gene from the sea urchin Lytechinus variegatus (25) was a generous gift of Dr. Robert Simpson. HeLa (S3 strain) cells were purchased from ATCC (American Type Culture Collection; Rockville, MD). Sephacryl S-1000 was obtained from Pharmacia Biotech Inc., and Hydroxylapatite BioGel HTP was from Bio-Rad.

**HeLa Cells**—HeLa cells (S3 strain) were grown in spinner culture at 37°C in the presence or absence of sodium butyrate as described elsewhere (14).

**Preparation of Histone Octamers**—Chicken erythrocyte histone octamers were obtained as described elsewhere (20). Control and highly hyperacetylated histone octamers from HeLa cells were prepared from chromatin fractions isolated as described previously (14). Only fraction a (14) was used as a source of hyperacetylated histones. The chromatin fraction (~6 mg) was then dialyzed against 0.633 M NaCl, 0.1 M potassium phosphate, 1 mM dithiothreitol (pH 6.7) with or without 5 mM sodium butyrate and loaded onto a hydroxylapatite column (1.8 × 15 cm at a flow rate of 16 ml/h) previously equilibrated with the same buffer. After elution of the linker histones under these conditions (about 100 ml) the eluting buffer was changed to 1 M NaCl in the same volume.
buffer to elute the histone octamers. The histone octamers obtained in this way were concentrated in a centrifuge 10 and used immediately thereafter or kept frozen at −60 °C.

Preparation of the 208–12 DNA Template—The 208–12 DNA template used in the oligonucleosome reconstitutions was prepared as described in Ref. 19.

Oligonucleosome Reconstitution—This procedure was carried out as described previously (19).

Gel Electrophoresis—SDS-15% polyacrylamide gel electrophoresis was carried out according to Laemmli (21). Acetic acid, 8 M urea, 0.5% Triton X-100, polyacrylamide gel electrophoresis was carried out according to Ref. 22 with a few minor modifications. The acrylamide:bisacrylamide ratio was 30:1 (separating gel) and 20:1 (stacking gel). The sample buffer was 10 M urea, 5% acetic acid, 5% β-mercaptoethanol, and 0.3% Pyronine Y.

1.2% agarose gels were prepared in TBE buffer (25 mM Tris borate, 0.5 mM EDTA, pH 8.0), according to Maniatis et al. (23).

Electron Microscopy—Preparation of the samples for electron microscopy was carried out according to Labhart and Koller (24) as described in Ref. 19. Micrographs were taken with a Philips 301 electron microscope.

Analytical Ultracentrifuge Characterization—Sedimentation velocity and sedimentation equilibrium analysis were performed on a Beckman model E analytical ultracentrifuge with a computer-interfaced UV scanner (Ultracentrifuge Interface and Data Analysis Program version 1.70; Borries Demeler (Missoula, MT). Experimental conditions and analysis of the runs were as described previously (19) or as indicated in the figure legends.

RESULTS

We have characterized in the past (19, 20) the folding behavior of reconstituted oligonucleosome complexes using a DNA template consisting of 12 tandemly arranged copies of a 208-base pair fragment of 5 S rRNA gene from the sea urchin *L. variegatus* and chicken erythrocyte core histones. In the present work we have reconstituted 208–12 oligonucleosome complexes with the same DNA template but using highly acetylated core histones obtained from HeLa cells grown in the presence of sodium butyrate (to inhibit the cell deacetylases) and core histones obtained from HeLa cells grown in the absence of butyrate (used as a control). To this purpose, the chromatin from the butyrate-treated cells was fractionated based on its differential solubility in the presence of divalent cations to yield fractions highly enriched in highly hyperacetylated core histones (14). Upon depletion of histone H1 the core histones from both butyrate (acetylated) and non-butyrate (control) treated cells were purified under nondenaturing conditions using hydroxylapatite as described under “Experimental Procedures.”

Fig. 1 shows the electrophoretic analysis of the core histones (panels A and B) as well as the DNA template and the resulting reconstituted oligonucleosome complexes (panel C).

The acetylated histones used in this work (Fig. 1A, lanes 1) correspond to fraction a of the chromatin fractionation procedure (14) and contain an average of ~17 acetyl groups/histone octamer (14). The core histones obtained from non-butyrate-treated HeLa cells are shown in Fig. 1A, lanes 2, and in Fig. 1B, lane 2. Fig. 1 also shows a native agarose gel of the oligonucleosome complexes reconstituted with hyperacetylated histones (panel C, lane A) and histones of low level acetylation (Fig. 1C, lane N) in comparison with the 208–12 DNA used as a template (Fig. 1C, lane D).

In agreement with previous observations (19, 25), the electrophoretic mobility of the 208–12 oligonucleosome complexes reconstituted with nonacetylated core histones is very similar to that of their 208–12 DNA template. Under the electrophoretic conditions used here (see “Experimental Procedures”) the acetylated oligonucleosome 12-mers also exhibit an almost identical mobility (Fig. 1C, lane A). Sedimentation equilibrium analysis carried out with both samples (see also Fig. 2) provided linear plots of log (absorbance) versus the square of the radial distance for the concentration gradient at equilibrium. The molecular weight established from these plots *M* = $2.92 \times 10^6 \text{ (nonacetylated oligonucleosome complexes)}$ and *M* = $2.93 \times 10^6 \text{ (acetylated oligonucleosome complexes)}$ is fully consistent with the presence of 12 nucleosomes/molecule of DNA template (19).

The salt-dependent hydrodynamic behavior of the control and hyperacetylated oligonucleosome complexes is shown in Fig. 3 in comparison with the hydrodynamic data obtained from oligonucleosomes reconstituted with chicken erythrocyte histones.

As can be seen in Fig. 3A, both the HeLa control oligonucleo-
somes (reconstituted with histones from non-butyrate-treated HeLa cells) and the oligonucleosomes reconstituted with chicken erythrocyte histones behave very similarly. As has already been exhaustively discussed (19), this dependence of the sedimentation coefficient within this ionic strength range (0–100 mM NaCl) corresponds to a folding pattern of the oligonucleosome structure such as that shown in Fig. 5B. Unfortunately, our results do not allow us to establish whether this folding is due to the bending of the internucleosomal DNA linker regions (26), to an additional wrapping of these DNA regions about the histone octamer, or to a combination of both effects. However, it is obvious that the increase in the salt within the 0–100 mM range provides charge screening of the phosphates in the DNA backbone that allows for adjacent nucleosomes to come together.

In contrast, oligonucleosomes reconstituted with histones from bytyrate-treated HeLa cells exhibit a completely different behavior. At low salt (10 mM Tris-HCl, 0.1 mM EDTA, 3 mM sodium butyrate, pH 7.5) the sedimentation coefficient of these complexes (~22 S) is much lower than that of the control counterparts (~27 S) for HeLa histone oligonucleosomes or 29 S for chicken erythrocyte-histone oligonucleosomes. Simple modeling calculations using the Kirkwood formalism (27) (see also Ref. 19 for a detailed analysis) suggest that such a decrease in S value can be accounted for by an unfolding of the oligonucleosome fiber as a result of a partial unraveling of the nucleosomal DNA coil at the flanking sides of the acetylated nucleosome particle. The model is fully consistent with earlier data on the melting properties of hyperacetylated nucleosome core particles (14, 28). It was shown that hyperacetylated nucleosome core particles exhibited a significant increase in the first melting transitions (14) corresponding to the DNA regions at the ends of the DNA supercoil (29, 30). Indeed, a release of the DNA affecting about 18.5 base pairs within these regions has been more recently proposed (31).

As the salt concentration increases, the sedimentation coefficient of the hyperacetylated oligonucleosome complexes also increases, reaching a plateau at about 100 mM NaCl with an $s_{20}$ = 29 S (Fig. 3B). This value is very similar to the sedimentation coefficient of the control oligonucleosomes at low salt (see Fig. 3A), in which the linker DNA regions are in a rather extended conformation. These hydrodynamic data clearly indicate that under ionic strength conditions similar to those found under physiological conditions and in the absence of histone H1 acetylated oligonucleosome fibers retain an extended conformation.

A similar change in conformation would also explain the slight shift observed in Fig. 3A for the curves corresponding to chicken erythrocyte oligonucleosomes and to hyperacetylated histone oligonucleosomes due to the differences in their basal levels of acetylation (0.6 acetyl residues/histone H4 for chicken erythrocytes and 1.2 acetyl residues/histone H4 in bytyrate-un-treated HeLa cells (32)).

Further experimental evidence in support of this comes from the electron microscopy analysis shown in Fig. 4. In contrast to what happens to nonacetylated oligonucleosome fibers (19), hyperacetylated oligonucleosomes remain in an unfolded conformation within the range of salt concentrations analyzed here. At low ionic strength (see Fig. 4, 0 mM) the fibers exhibit an extended conformation that is reminiscent of that exhibited by trypsinized oligonucleosomes (19) and agrees well with the hydrodynamic data.

**DISCUSSION**

The results presented in the previous section conclusively show that histone acetylation has a positive role in the folding
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dynamics of the chromatin fiber. In the absence of linker histones under ionic strength conditions, similar to those of the physiological environment, acetylated polymnucleosomal fibers remain in an extended conformation in contrast to their non-acetylated counterpart. These results suggest that, at the chromatin level, the major structural effect of the acetylation of the N-terminal regions of the histones is on the linker DNA connecting adjacent nucleosome particle. This is fully consistent with the finding that histone acetylation reduces the nucleosome core particle linking number change previously reported by Norton et al. (31). It is also in good agreement with our earlier observation that the linker region is attacked more readily by micrococcal nuclease in hyperacetylated stripped chromatin (33).

Nevertheless, all these data are in apparent contradiction with the results recently obtained with SV40 minichromosomes from viral particles isolated from butyrate-treated cells (34). Although the histones of the SV40 minichromosomes exhibited a high extent of acetylation, no reduction in the level of constrained supercoiling could be detected (34).

In an attempt to explain these experimental discrepancies it has been argued (34) that whereas in the in vivo systems histone acetylation occurs in nucleosomes that are already assembled, in the in vitro oligonucleosome systems used by Norton et al. (31) oligonucleosomes were assembled (reconstituted) from histones that were already acetylated. However, this explanation seems very unlikely for the following reasons. 1) Our previously reported enhanced nuclease accessibility of the linker regions (33) was observed in a native (nonreconstituted) chromatin. 2) As will be discussed next, some of the structural effects of histone acetylation on chromatin folding can be mimicked "in vitro" (to a certain extent) by histone trypsinization (removal of the histone N-terminal domains by trypsin). It was shown, in this case, that the same structural effects can be observed regardless of whether in situ trypsinized oligonucleosomes or oligonucleosomes reconstituted from trypsinized core histones were used (19). 3) It was also shown that the reconstituted 208–12 oligonucleosome system has a folding behavior identical to that of "native" oligonucleosomes isolated from chicken erythrocyte chromatin (19). This also argues against any DNA sequence specificity as being responsible (34) for the results retained with the hyperacetylated oligonucleosome construct used by Norton et al. (31) and which is very similar to that used in this paper.

The predominant effect of histone acetylation on the linker DNA region also explains the larger conformation effect observed at the nucleosome level (16, 35) when compared with the core particle (14).

The results obtained here with the hyperacetylated oligonucleosome complexes together with our earlier studies with native and reconstituted trypsinized oligonucleosomes provide experimental support to the multistate model of chromatin folding (1) (see Fig. 5).

Although the role of the core histone tails in chromatin folding, has been long recognized (36, 37), these regions seem to play a much more important role in the dynamic modulation of this folding than was initially envisaged. In the absence of H1, the interactions of the core histone tails with the linker DNA regions provide the charge neutralization necessary to allow, under physiological conditions, the bending (or additional wrapping about the histone octamer) of this DNA region. This brings adjacent nucleosome particles together in a partially folded intermediate order state (IOS).1 It is clear from all our studies that under ionic strength conditions similar to those found in the cell nucleus, chromatin depleted of linker histones does not exhibit an extended conformation unless the histones are hyperacetylated. For simplicity, we have represented this intermediate state of folding as one in which nucleosomes are arranged in a contacting bidimensional zigzag organization, which is consistent with simple hydrodynamic modeling calculations (19, 20). Nevertheless the possibility of more complex three-dimensional arrangements arising from some kind of helical folding cannot be excluded (38).

This IOS structure is not easily amenable to transcription (39). Transcription may be favored upon transition to LOS modulated by histone acetylation. In fact both transcription initiation and elongation by RNA polymerase III have been shown to be significantly inhibited by the LOS → IOS transition (39).

In the presence of linker histones the extent of compaction of the chromatin fiber increases dramatically giving rise to the higher order structure (HOS) organization. Early studies on the effect of histone acetylation on the HOS of the chromatin fiber showed an almost negligible effect (17, 18). Therefore, the biological relevance of our finding has to do with the still unsolved issue of the linker histone stoichiometry in transcrip-

1 The abbreviations used are: IOS, intermediate order state; HOS, higher order structure; LOS, low order structure.
Fig. 5. Schematic representation of the dynamic transitions of chromatin. A, "textbook" representation of the histone H1 modulation of chromatin structure. This represents the currently accepted model for the transition of the higher order structure (HOS, 30-nm fiber) to the low order structure (LOS) of chromatin. According to this model, depletion (displacement) of histone H1 in vivo (by phenomena still not known), leads to an unfolding from HOS to LOS. This extended LOS conformation of chromatin would allow for the mechanisms of recognition of specific DNA sequences by activating factors and would eventually allow for chromatin activation (transcription, replication). B, multistate folding of chromatin structure (1). This is our model based on the experimental results presented in this paper and in Ref. 19. According to this model, upon removal/displacement of histone H1, under physiological "native" conditions, the chromatin fiber only partially unfolds, thus remaining in intermediate folded conformation (intermediate order structure, IOS). Complete unfolding of this IOS chromatin fiber into a completely unfolded conformation (LOS) is only achieved by lowering the concentration of DNA counters, either by decreasing the ionic strength or by removing the highly cationic domains (histone tails) of the core histones by trypsinization. We have recently provided thorough and detailed evidence for this model (19). C, based on the model of panel B, a hypothesis is made, and experimentally supported in this paper, that a HOS → LOS transition upon histone H1 depletion is possibly modulated in vivo by acetylation of the histone octamers through a physical counterion mechanism virtually identical to that described in panel B. Acetylated chromatin in this LOS conformation would thus become available for specific DNA sequence recognition and binding of activating factors (45) and/or to allow transcriptional elongation by polymerases.

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REFERENCES
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