# Role of H1 in Chromatin Folding. A Thermodynamic Study of Chromatin Reconstitution by Differential Scanning Calorimetry†

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ABSTRACT: In a series of related papers, we have recently presented the results of a thermodynamic approach to the conformational transitions of bulk chromatin induced *in vitro* by different structure-perturbing agents, such as the intercalating dye ethidium bromide or the ionic strength. In all these studies, we took advantage of the capability of differential scanning calorimetry to detect the changes in the melting behavior of the structural domains of chromatin (the linker and the core particle) associated with the order−disorder transitions. This technique also revealed that the higher-order structure undergoes a catastrophic decondensation process in the course of the transformation of rat hepatocytes as well as of cultured cells. Therefore, several questions arose concerning the biological function (if any) of the changes in the degree of condensation of bulk chromatin, as well as the mechanism of transition and the nature of the modulating agents. In this paper, we report a thermodynamic analysis of the reconstitution of H1-depleted calf thymus chromatin with the purpose of establishing (1) the binding mode of H1 and (2) the energetics and cooperativity of the transition from the unfolded to the condensed state. When H1 is progressively extracted from calf thymus nuclei by high-salt treatment, the endotherm at 107 °C, characteristic of the core particles interacting within condensed domains, converts into the thermal transition at 90 °C, resulting from the denaturation of noninteracting core particles. Binding of H1 fully restores the thermal profile of native chromatin. Analysis of H1 association shows that binding occurs at independent sites with $K_A = (3.67 \pm 0.60) \times 10^4\ M^{-1}$ and each site comprising 180 ± 10 bp. The experimental dependence of the fraction of condensed chromatin on $R$, the moles of bound H1 per nucleosome mole, was compared with a simple thermodynamic model for the conformational change. This analysis yields a value of −5 kcal per nucleosome mole for the interaction free energy of nucleosomes within the ordered state. The process of condensation, is not, however, a highly cooperative (all-or-none) one, as expected from a consideration of the solenoidal model for the 30 nm fiber. Rather, nucleation of the helical state involves the face-to-face interaction between consecutive core particles, and the growth is largely determined by the mergence and rearrangement of neighboring clusters of helically arrayed nucleosomes.

In a brilliant note to the paper of Reuter et al. (1990) on the modulation of position−effect variegation in *Drosophila*, Alberts and Sternglanz (1990) have renewed interest in an early hypothesis on the biological function and inheritance of the higher-order structure of chromatin. What is particularly suggestive in the model, which substantially goes back to 20 years ago (Cook, 1973; Alberts et al., 1977), is the corollary according to which the inheritance of the pattern of gene expression through cell lineages depends on structural and thermodynamic determinants; namely, a nucleation mechanism is postulated which warrants the exact duplication of the superstructure of the genome during S phase. Thus, the propagation of the functional state of a gene is committed to a definite "crystalline" array of nucleosomes and non-histone proteins, corresponding to a local minimum in the conformational free energy along the chromatin strand. Specific interactions among different DNA sequences, histone variants, and proteins of the nuclear scaffold are expected to modulate the nucleation process.

Whenever one considers heuristic models for permanent gene repression, one immediately recognizes a remarkable lack of information, even about the basic parameters of the process of condensation, as the free energy changes and cooperativity. In position−effect variegation, for example, inactivation can involve the spreading of heterochromatin along the DNA for enormous distances from the site of the chromosome rearrangement (Reuter et al., 1990), a crystallization event amenable to direct thermodynamic analysis. However, we have at present no means to verify this hypothesis from the molecular standpoint.

Perhaps because of the emergence of new questions from molecular biology, a few papers have been recently devoted to the thermodynamics of compaction of both H1-containing (Yao et al., 1990) and H1-depleted (Yao et al., 1991; Garcia-Ramirez et al., 1992) chromatin oligomers, as well as to the characterization of the conformation and dynamics of H1-

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depleted chromatin (Hansen & Ausio, 1992), regarded as a model system for transcriptionally active genes. At physiological ionic strength, dinucleosomes assume a compact conformation; the linker DNA sharply bends, bringing the core particles into contact (Yao et al., 1990). In a subsequent paper, Yao et al. (1991) showed that compaction is not abolished when H1 is removed, and argued that the histone is not essential for chromatin condensation. As a fact, the driving force of the process resides to a large extent in the $NH_2$-terminal region of the core histones, as demonstrated by selective trypsinization of this domain (Garcia-Ramirez et al., 1992). These important findings clearly implicate that several views on the mechanism of condensation must be critically reexamined. If consecutive nucleosomes are able to interact strongly in the 30 nm fiber, then the nucleation of the ordered state could turn out to be at variance with the one deducible from the geometry of current structural models. For example, the solenoid accommodates up to six nucleosomes per turn; major stabilizing interactions arise from the edge-to-edge pairing of nonconsecutive nucleosomes (Widom & Klug, 1985). Moreover, since H1-depleted chromatin assumes a compact (zig-zag) conformation at physiological ionic strength (Garcia-Ramirez et al., 1992), the role of the histone in condensation must be more sophisticated than believed until now, as an ordered array of nucleosomes, rather than a chain in a random conformation, undergoes the transition to a higher level of folding. A direct answer to all of these questions can be in principle obtained by the detailed thermodynamic analysis of the reconstitution of H1-depleted nuclear chromatin. Such a study is reported in the present paper.

A previous investigation on the folding mode of reconstituted chromatin relied on the sedimentation analysis of complexes formed at different ratios of H1 to nucleosome; complementary information on the compaction of the polynucleosomal chain was obtained by electron microscopy (Allan et al., 1981). This approach succeeded in demonstrating the progressive increase in the packing ratio of the fibers with increasing amount of bound H1. The dependence of the sedimentation coefficient on this parameter was, however, almost linear, suggesting that the linker histone does not bind cooperatively to stripped polynucleosomes. In contrast, Watanabe (1984) present experiments of fluorescence titration of labeled H1 pointing to strong cooperativity. Even if this remarkable disagreement is ignored, the severe limitations of the methods currently applied in the study of chromatin condensation should be clearly borne in mind. For such investigations to be informative, the experimental dependence of the fraction of nucleosomes packed in the higher-order structure on the amount of bound H1 must be determined, but, of course, the former quantity cannot be obtained by electron microscopy and sedimentation experiments, which only afford a measure of the average contraction ratio of the fiber.

It has been recognized early that the melting behavior of chromatin, due to its sharp subdivision into energetically distinguishable domains, holds detailed structural information. Optical melting methods have been successfully used for characterizing the conformational transitions of isolated chromatin (Fulmer & Fasman, 1979a) as well as the reconstitution of H1-stripped polynucleosomes with the phosphorylated histone (Kaplan et al., 1984). The extension of this approach to chromatin at physiological ionic strength, where optical techniques can no longer be used to monitor the denaturation process, has been pursued in our laboratory over recent years. The differential scanning calorimetry (DSC)[1] profile of isolated chromatin and nuclei shows several thermal transitions which reflect the denaturation of structural domains (Nicolini et al., 1983). The molecular assignment of each endotherm has been reported in a series of related papers (Balbi et al., 1988, 1989; Cavazza et al., 1991). In a recent study on the chromatin changes which occur in transformed cells (Barboro et al., 1993), we have outlined a concise account of the major outcomes of the DSC method. Here, it is sufficient to recall that the core particle DNA melts at ~90 °C when the core particles do not interact, but the denaturation temperature shifts to 107 °C when nucleosomes interact within ordered (condensed) domains (Cavazza et al., 1991). The determination of the fraction of packed nucleosomes rests essentially on this thermodynamic feature of the condensation.

In the present analysis of chromatin reconstitution, undigested, H1-depleted nuclei were used as the starting material. In this way, several constraints, which are believed to be involved in the assembly of chromatin *in vivo*, such as the anchorage of DNA to the nuclear scaffold, have been maintained in the *in vitro* experiments.

## EXPERIMENTAL PROCEDURES

*Preparation of H1-Depleted Calf Thymus Nuclei.* Nuclei were isolated from calf thymus, as we already described (Cavazza et al., 1991), except that all the buffers were supplemented with a mixture of inhibitors of proteases (20 $\mu$g/mL leupeptin, 25 $\mu$g/mL aprotinin, 10 $\mu$g/mL peptstatin, and 0.5 mM benzamidine) in addition to 5 mM $Na_2S_2O_5$ and 1 mM phenylmethanesulfonyl fluoride (Barboro et al., 1993). Histone H1 was removed at 4 °C in the presence of inhibitors using 24 mM $Na_2EDTA$, pH 7.8, containing 0.6 M NaCl as the extraction buffer. Weighed amounts of pelleted nuclei (50−100 mg) were added to test tubes containing a large excess (10 mL) of extraction buffer, and the dissociation of H1 was allowed to proceed without stirring. After 1 h incubation, the supernatant was removed by gentle sucking with a Pasteur pipet and another aliquot of fresh buffer added. This step was repeated several times; as a rule, a 24 h extraction was needed in order to completely remove the histone. For the purpose of characterizing the time course of both the residual H1 content and the nucleosome spacing, the nuclear pellet was repeatedly washed with extraction buffer and digested with micrococcal nuclease or used for the extraction of the histone complement as described in the following.

*Preparation of H1.* Calf thymus H1 was prepared from isolated nuclei according to the first method of Johns (1964). The preparation contained small amounts (5−7% by weight) of low molecular weight contaminants including HMG 1 and HMG 2 and was purified by chromatography on Bio-Gel P-100 as described by Nelson et al. (1979); a 2.6 × 85 cm column, operating at a flow rate of 0.1 mL·min$^{-1}$, was used; the elution profile was monitored by recording the absorbance at 278 nm. The peak elution volume of H1 was about

[1] Abbreviations: DSC, differential scanning calorimetry; $Na_2EDTA$, ethylenediaminetetraacetic acid disodium salt; SDS, sodium dodecyl sulfate; DM, dissociation medium; Tris, tris(hydroxymethyl)aminomethane; mol bp, moles of base pairs.

150 mL. Five milliliter fractions, corresponding to the central part of the peak, were electrophoresed on 14% SDS–polyacrylamide gels, and those containing highly purified H1 were pooled, concentrated in an Amicon Model 8200 cell, dialyzed against distilled water, and lyophilized.

*Reconstitution of Chromatin.* In order to establish whether kinetic factors affect the properties of reconstituted chromatin, the experiments were carried out by both continuous flow salt gradient dialysis (Carroll, 1971; Kaplan et al., 1984) and direct mixing (Allan et al., 1981). In the former experiments, appropriate amounts of extraction buffer and of a concentrated solution (5–10 mg/mL) of H1 in the same solvent were added to the pellets of H1-depleted chromatin, up to a final volume of 3 mL; the samples were transferred to dialysis bags under gentle shaking. Reconstitution was then carried out at 4 °C employing the experimental setup shown in Figure 1b of Carroll's paper (Carroll, 1971); vessel 1 contained 500 mL of extraction buffer, and vessel 2 2 L of DM, consisting of 75 mM NaCl and 24 mM $Na_2EDTA$, pH 7.8. The flow was driven by a peristaltic pump, operating at a flow rate of either 100 (slow dialysis) or 500 mL·h$^{-1}$ (fast dialysis). After a final (1 h) dialysis against DM, which was required in order to equilibrate the samples with the buffer, reconstituted nuclei were recovered by centrifugation at 10000*g* and transferred to calorimetric capsules, as already described (Cavazza et al., 1991). For determination of the concentration of both DNA and bound H1, the samples were centrifuged at 37000*g* and briefly washed with an excess of DM, in order to remove the residual free histone. The same procedure was followed to prepare the material submitted to digestion with micrococcal nuclease.

*Analytical Techniques.* The histone complement was extracted from all the nuclear samples according to the method of Panyim et al. (1971). Prior to the precipitation of histones with ethanol, the DNA was recovered by centrifugation at 12500*g*. Histones were run on 14% SDS–polyacrylamide gels according to Laemmli (1979). The gels were stained with 0.1% Amido black in 35% acetic acid and 20% methanol and destained in 7% acetic acid containing 1 mg/mL Amido black, and the amount of H1 was determined by scanning at 633 nm in the LKB UltroScan XL densitometer Model 2222-010; the scans of the electrophoretograms of known amounts (from 5 to 40 $\mu$g) of H1 were used to generate a calibration curve. The reproducibility of the color yield was checked in all the determinations by electrophoresing 10 and 40 $\mu$g of H1 in parallel with the extracted histones. The quantitation of H1 by Amido black staining is reasonably accurate (limits of the standard deviation ±6%) as already shown by Panyim and Chalkley (1969) and compares favorably with the procedures involving the use of Coomassie brilliant blue, since we have verified that the latter dye yields a nonlinear dependence of the color response on the amount of H1 electrophoresed. However, for a rapid evaluation of the amount of residual H1 in the course of the preparation of H1-depleted nuclei, the gels were stained with 0.1% Coomassie brilliant blue in 10% acetic acid and 45% methanol and destained with 5% acetic acid and 20% methanol, and the content of H1 was determined by scanning the gels as above, using the core histones as an internal standard. For the quantitation of DNA, the pellet isolated prior to the precipitation of histones was dissolved in 40 mL of 0.1 N NaOH, and the concentration was determined spectrophotometrically at 260 nm assuming an extinction coefficient $E^{1\%} = 300$ for alkali-denatured DNA. This rapid and direct procedure was proven to be very reliable, as judged by comparison with the results obtained by the current method for the isolation of genomic DNA (Maniatis et al., 1982).

For the determination of $K_A$, the intrinsic association constant of H1 to H1-depleted chromatin, and $n$, the number of binding sites per base pair, the equation for binding at independent sites was fitted to the data, cast in the form of a saturation plot of $r$ vs $C_f$, where $r$ is the moles of H1 bound per mole base pairs and $C_f$ the molar concentration of free H1, using the Enzfitter program on an IBM PC; $C_f$ was calculated by the difference from the concentrations of total and bound H1.

*Circular Dichroism Measurements.* Circular dichroism spectra of H1 were recorded at 23 °C in a Jasco Model J 500-A spectropolarimeter in the concentration range from 0.1 to 0.5 mg/mL using 0.1 cm path length cells. Due to the high absorbance of NaCl in the far-ultraviolet region, the secondary structure of H1 in the extraction buffer was estimated assuming that all the monovalent salts exert comparable effects on the conformation. Therefore, the CD spectrum was determined in 20 mM Tris, pH 8, containing 0.6 M KF, which has good transparency below ~220 nm (Adler et al., 1973). The data were expressed in terms of $[\theta]$, the mean residue ellipticity in units of degrees centimeter squared for decimole of residue; a mean residue molecular weight of 100.8 was calculated from the amino acid composition of calf thymus H1 reported by Böhm et al. (1982). The $\alpha$ helix, $\beta$ sheet, and "random coil" contents were calculated by analyzing the CD curves according to the method of Greenfield et al. (1967); the $\alpha$ and $\beta$ sheet spectra of poly(L-lysine) (Greenfield & Fasman, 1969) and the circular dichroism curve of unfolded H1 in 0.01 N HCl determined in this work were taken as the basis in the computation.

*Electron Microscopy of H1-Depleted Chromatin.* Pelleted H1-depleted nuclei (~30 mg) were resuspended in 1 mL of digestion buffer (75 mM NaCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, and 10 mM Tris, pH 7) and mildly digested with 15 units/mL micrococcal nuclease (Sigma) for 1 min at 37 °C. Soluble chromatin was extracted with 0.2 mM $Na_2EDTA$, pH 7, and mounted for electron microscopy on carbon-coated grids according to the (benzyldimethylalkyl)ammonium chloride technique (Sogo & Thoma, 1989). The specimens were rotatory-shadowed with platinum at an angle of 16° and observed in a Siemens 102 electron microscope operating at 80 kV.

*Other Methods.* For analysis of nucleosome spacing, native, H1-depleted, and reconstituted nuclei were resuspended in digestion buffer as described above and digested at 37 °C with 20, 2, and 20 units/mL micrococcal nuclease for 20 min, respectively, and the DNA was isolated according to a standard procedure (Maniatis et al., 1982). The electrophoresis of DNA on 0.6% agarose gels was performed as reported in a previous paper (Cavazza et al., 1991). Densitometric scanning of the gels was carried out on a Kontron Elektronic Vidas 2.1 image analysis system. DSC of the nuclear pellets in DM was carried out as already described (Balbi et al., 1989; Cavazza et al., 1991).

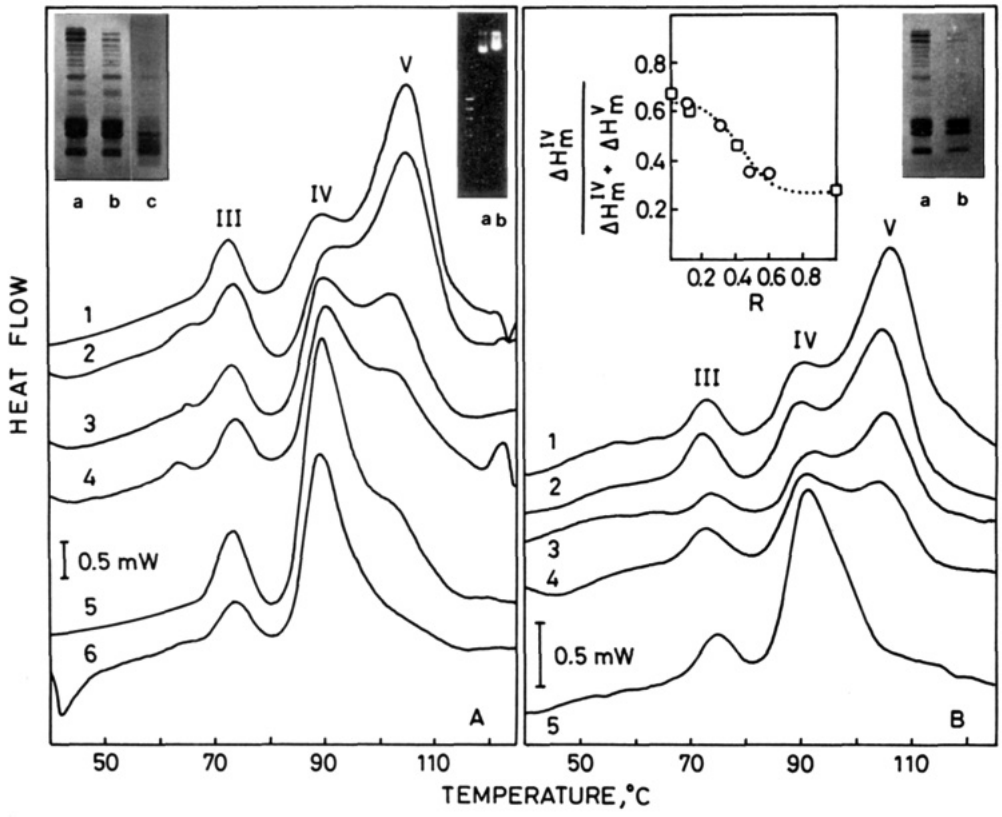


FIGURE 1: Progressive changes in the thermal profile of calf thymus nuclei induced by digestion (A) or extraction (B) of H1. The transitions of the DNA domains are marked by Roman numerals. (A) Nuclei incubated in DM at 4 °C for different times. (1) Control nuclei; (2) 3 h; (3) 24 h; (4) 48 h; (5) 8 days; (6) 15 days. Left inset: 14% SDS−polyacrylamide gel electrophoresis of the histone complement isolated from the control (lane a) and from nuclei incubated for 24 and 48 h, respectively (lanes b and c). Right inset: Agarose gel electrophoretic pattern of the DNA extracted from the control (lane a) and from nuclei incubation for 48 h (lane b). The size marker is ΦX174/*Hae*III fragments. (B) Extraction of H1 with 25 mM $Na_2EDTA$ containing 0.6 M NaCl. (1) Control nuclei; (2−5) nuclei treated with the extraction buffer for (2) 30 min, (3) 2 h, (4) 8 h, and (5) 24 h. The right inset reports the electrophoretic characterization of the histones isolated from the control (lane a) and from nuclei treated with the extraction buffer for 24 h. Note that at this time H1 has been almost completely removed. The left inset shows the dependence of the fraction of decondensed chromatin, given by $\Delta H^{IV}_m/(\Delta H^{IV}_m + \Delta H^{V}_m)$, on $R$, the moles of bound H1 per nucleosome mole. Digestion and extraction experiments are marked with squares and circles, respectively. The dependence of $\Delta H^{IV}_m/(\Delta H^{IV}_m + \Delta H^{V}_m)$ on $R$ determined in the reconstitution experiments (see Figure 5A) is also shown for comparison (•••).

## RESULTS AND DISCUSSION

*H1 Digestion or Its Extraction Result in the Conversion of the 107 into the 90 °C Endotherm.* H1 removal represents a sufficient condition for chromatin decondensation, and consequently we shall show first that when the linker histone is either digested by endogenous proteases or extracted using a high-salt buffer, endotherm V at 107 °C, arising from the denaturation of tightly packed core particles, is converted into thermal transition IV at 90 °C, characteristic of the unfolded state of chromatin (Balbi et al., 1989; Cavazza et al., 1991). In other words, we shall verify that H1-depleted chromatin [referred to here as core chromatin following Fulmer and Fasman (1979a)] at physiological ionic strength shares the major features of the DSC profile with whole chromatin unfolded in low-salt buffers (Cavazza et al., 1991).

Although the thermal profile of calf thymus nuclei stored in DM at 4 °C for several days undergoes as a rule only a limited drift in the enthalpies of the high-temperature transitions (Cavazza et al., 1991), we recently noticed that cells isolated from animals showing severe thymus involution, quite likely as a consequence of prolonged treatment with glucocorticoids (Betta et al., 1990), were characterized by high levels of a histone-specific protease, that results both in H1 digestion and in loss of transition V. This observation, although empirical, shows in a straightforward way the actual dependence of chromatin condensation on H1 content. A typical result is reported in Figure 1A. After a 24 h incubation in DM at 4 °C (scan 3), the enthalpy of the 107 °C endotherm, which is dominant in the thermogram of native nuclei (scan 1) is to a large extent transferred into transition IV. The inset shows 14% SDS−polyacrylamide gel electrophoresis of the histone complement isolated from the control (lane a) and from nuclei incubated for 24 h (lane b). A strong decrease in the content of H1 is apparent; the corresponding values of $R$, the moles of bound H1 per nucleosome mole, determined by densitometry of the Coomassie brilliant blue stained gel are 1 and 0.45, respectively. Complete decondensation is attained after 15 days (scan 6); after 48 h, however, the process is near completion, since the high-temperature region of the thermal profile shows nothing but a small shoulder at about 100 °C in the 90 °C transition. At this time, H1 is almost completely digested (lane c), and also the core histones begin to be cleaved. This correlation between the decrease in $\Delta H^{V}_m$ and digestion of H1 does not require further comments. The electrophoretic patterns of the DNA isolated from native material and nuclei incubated for 48 h are compared in the inset. No difference in the migration can be detected; neither sample enters significantly in the agarose gel, showing that DNA chain scission is not involved in chromatin unfolding, at variance

with rat liver nuclei (Balbi et al., 1989). The pattern of degradation of histones outlined above is in line with previous observations on a general proteolytic activity associated with calf thymus chromatin (Furlan & Jericijo, 1967). It is worth noting that the protease is almost completely removed by repeated extractions with buffers containing high concentrations (above 0.35 M) of NaCl, since this treatment restores both the stability of the histone complement and the steadiness of the thermal profile in the incubation experiments (Balbi et al., unpublished results).

When H1 is progressively extracted using 25 mM $Na_2$-EDTA containing 0.6 M NaCl (Figure 1B), the changes induced in the thermal profile match with those arising from H1 digestion. This result is shown diagrammatically in the inset, which reports the dependence of $\Delta H^{IV}_m/(\Delta H^{IV}_m + \Delta H^{V}_m)$ on $R$ for both protease-digested (squares) and salt-extracted nuclei (circles). The two sets of experimental values of this parameter, which represents a measure of the extent of unfolding (Barboro et al., 1993), follow the same dependence on $R$. In addition, they are in good agreement with the trend determined in the more accurate reconstitution experiments (dotted line), to be discussed in another section. Therefore, we can rule out that hysteresis phenomena affect significantly the conformation of chromatin in the course of H1 dissociation and reassociation.

*Preservation of the 190 Base Pair Repeat in Core Chromatin Prepared by High-Salt Extraction.* The employment of high-salt buffers to dissociate chromatin presents several advantages; no irreversible conformational changes of DNA and histones are induced, and, furthermore, the three-dimensional architecture and protein composition of the nuclear scaffold do not undergo gross alterations, as judged by ultrastructural and biochemical criteria. On the other hand, elevated ionic strength weakens the DNA–histone interactions, and promotes the motion of cores in H1-depleted chromatin (van Holde, 1989). If the energy barriers opposing long-range motion are abolished, sliding of the octamers can be observed, a process which reaches its height with the formation of "compact" oligomers (Steinmetz et al., 1978; Tatchell & van Holde, 1978; Spadafora et al., 1979). The tight association of the cores is directly reflected in the chain length distribution of the DNA fragments generated by micrococcal nuclease digestion of chromatin; the native 190 bp repeat is observed only in the early stages of digestion and converts, with increasing digestion time, into a 140 bp register (Steinmetz et al., 1978). In this section, we shall document that the native repeat is preserved in the material employed in the reconstitution experiments. However, sliding was observed whenever core chromatin experienced frictional forces, as reported by Steinmetz et al. (1979); if the extraction of H1 is carried out at 4 °C under vigorous stirring, or shaking, the formation of compact oligomers is induced. The characterization of this form of chromatin is beyond the aim of this work, and we shall limit to point out briefly the major structural differences with respect to the native state.

Figure 2A reports the electrophoretic patterns of the DNA isolated from native (lane a), partially H1-depleted (lanes b and c), and H1-depleted (lane d) nuclei; the digestion conditions were adjusted in order to attain comparable extents of DNA chain scission. It is apparent that the prolonged incubation in the extraction buffer does not affect the repeat length; after 24 h, when the histone is quantitatively removed, the densitometric scan of the gel (scan d) is still in register with the one of the control (scan a). The repeat length was determined from several experiments according to the method of Watkins and Smerdom (1985) which yields accurate values for great extents of digestion, when only a few bands are visible on the gel. The values for native and core chromatin were found to be 194 ± 5 and 191 ± 10 bp (mean of 10 determinations ± standard deviation), respectively, and are in excellent agreement with the value reported by Weischet et al. (1979) for the same materials (192 ± 4 bp). It has to also be noted that the native repeat is preserved in reconstituted chromatin as well (lane and scan f); at saturation ($R = 1$), we obtain 195 ± 11 bp, which is coincident within error with that of the control.[2]

An example of the altered periodicity which is induced when friction is exerted on chromatin in the course of the extraction of H1 is shown in Figure 2B, lane b; the monomer, dimer, trimer, and tetramer bands are centered at 146, 270, 360, and 563 bp. Therefore, this pattern conforms to the series of "most compact" oligomers (Tatchell & van Holde, 1978); the DNA content of each member is given by the expression $120n + 20$, where $n$ denotes the number of cores. The closely stacked cores can be easily identified in the electron micrographs as elongated or roundish particles separated by stretches of naked DNA (Figure 2D), and appear to be stabilized toward electrostatic repulsion, since they do not undergo unfolding in low ionic strength buffers, which induce the complete extension and opening of H1-stripped polynucleosomes (Figure 2C). The characterization of the denaturation behavior of compact oligomers is in progress in our laboratory. Transition III, corresponding to the unstacking of linker DNA, disappears, while a low-enthalpy, reproducible exotherm comes out at 72 °C. Melting of core particle and free DNA occurs in a complex series of endotherms above 85 °C (Balbi et al., unpublished results).

*Binding of H1 Core Chromatin Occurs at Independent Sites. Relation of the Present Analysis to Previous Reconstitution Studies.* The present knowledge of the mode of binding of H1 to core chromatin is essentially of a qualitative nature; as far as we know, only one paper (Watanabe, 1984) has reported a quantitative analysis of the binding equilibrium, based on fluorescence measurements. The process was found to be highly cooperative, with an association constant $K_A$ of $7.5 \times 10^7$ $M^{-1}$. We were unable to confirm this result. Indeed, analysis of the reconstitution data shows that H1 binding occurs in a noncooperative fashion, with a lower $K_A$. As we shall discuss in the following, this result is in line with well-established phenomenological aspects of H1-induced chromatin folding. On the other hand, scrutiny of the work of Watanabe (1984) shows that the reconstitution method employed was simply inadequate for preserving the nucleosome repeat.

It must be pointed out that, within a given set of experimental results concerning the binding of H1 to core

[2] It has to be remarked that the electrophoretic tracks of the DNA isolated from the digests of H1-depleted and reconstituted nuclei are not as sharp as those obtained from the control. This suggests that the treatment with 0.6 M NaCl induces a limited displacement of a fraction of the cores from the positions occupied in the native configuration. We are currently attempting to test whether condensation of reconstituted chromatin originates a quantized set of linker lengths, as predictable by the theoretical model of Widom (1992) for the twist constraints on linker DNA in the 30 nm fiber.

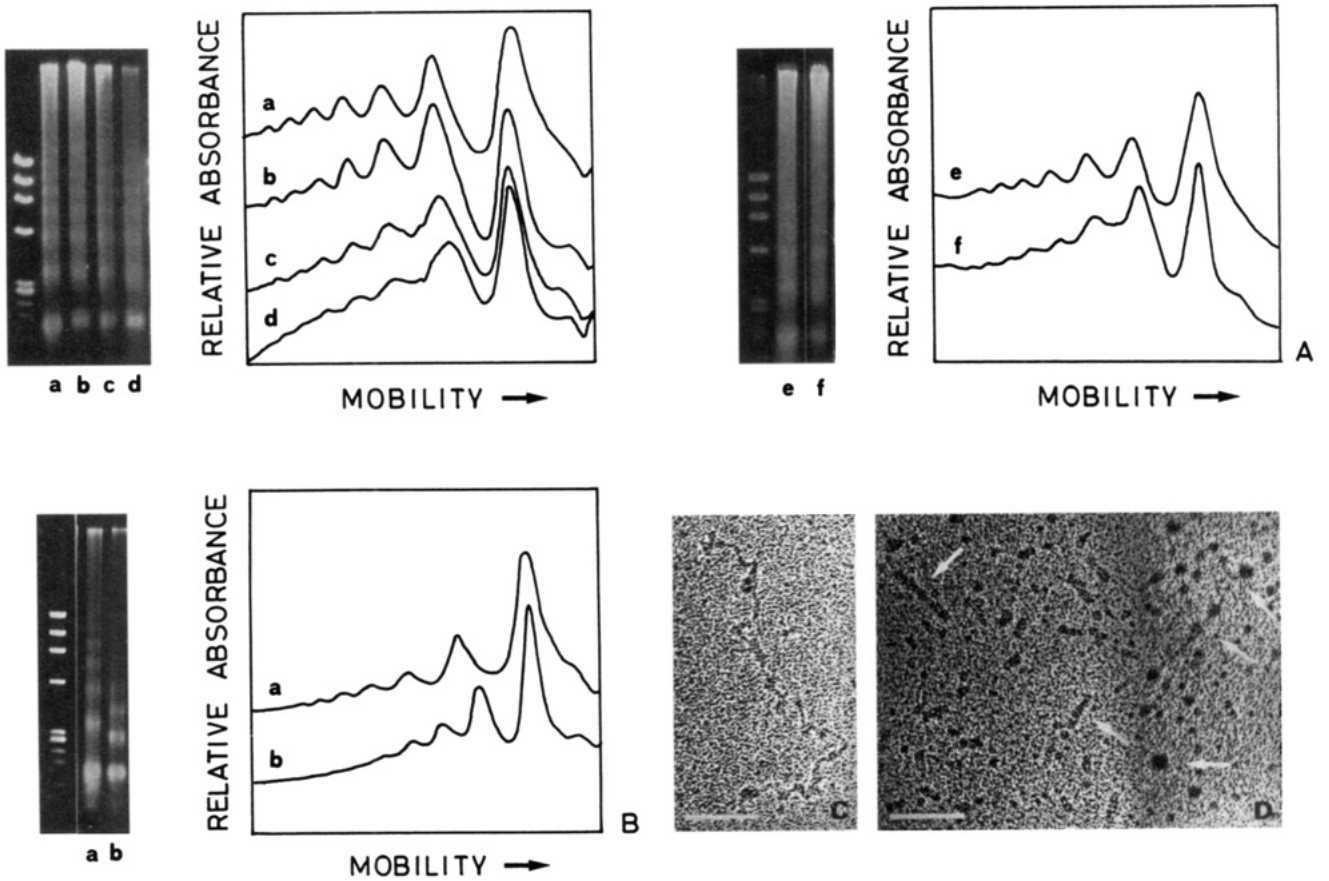


FIGURE 2: (A) Fragment lengths produced by micrococcal nuclease digestion of nuclear chromatin in the course of H1 extraction and after reconstitution. Lane a, control nuclei; lanes b, c, and d, nuclei extracted for 2, 4, and 24 h, respectively; lane e, control nuclei; lane f, digest of the reconstituted complex ($R = 1$). The corresponding densitometric tracings are marked by the same letter. (B) Electrophoretic comparison of the digests of H1-depleted nuclei prepared under stirring (lane b) and of control nuclei (lane a). The size marker is as in Figure 1. (C, D) Electron micrographs of H1-depleted chromatin in 0.2 mM $Na_2EDTA$ exhibiting native (C) and altered (D) repeat length. Note that in (C) most of the nucleosomes have been unfolded. Compact oligomers are marked by arrows in (D). The bar is 0.1 $\mu$m.

chromatin, the conformation of the histone may represent an additional parameter and must, therefore, be invariably characterized. Using microcalorimetry and determinations of the ellipticity at 222 nm as a function of the temperature, Crane-Robinson and Privalov (1983) have shown that the only folded domain of the molecule, corresponding to the central region between residues 36 and 121, is fully compacted in 50 mM Tris, pH 8, containing 0.2 M $K_2SO_4$. On the other hand, Smerdon and Isenberg (1976) have reported that the fractional change in folding of H1 subfractions, determined by fluorescence and CD, steadily increases above 0.2 M NaCl. The above note of caution applies, of course, to our experiments, since chromatin was reconstituted both by direct mixing in DM, at almost physiological ionic strength, and by gradient dialysis starting from the extraction buffer which contains 0.6 M NaCl. In the inset of Figure 3, we report the CD spectra of H1 in 0.6 M KF (full line), DM (dashed line), and 0.01 N HCl (dotted line); the latter was taken as the reference for the random coil state, and is shown for comparison. The values of the $\alpha$ helix, $\beta$ sheet, and random coil contents, calculated according to Greenfield et al. (1967), are 6, 4, and 89% in the case of the 0.6 M KF buffer and 7, 4, and 89%, respectively, when the determination is carried out in DM. Therefore, the experimental conditions of reconstitution adopted here do not involve appreciable differences in the structure of the histone, at least at the level of the secondary one. Barbero et al. (1980) have shown that the conformational transition of calf thymus H1 is complete at pH 10 in water, or in the presence of 0.3 M KF at pH 6. The contributions of $\alpha$ helix, $\beta$ sheet, and random coil to the secondary structure were 8, 3, and 89%. The agreement with the values determined in this work is good, and confirms that in our experiments the central domain is fully folded. These results, taken together, cannot, therefore, be reconciled with the occurrence of an increase in the secondary structure of the H1 subfractions above 0.2 M as reported by Smerdon and Isenberg (1976). Indeed, the values of the $\beta$ sheet content, obtained by these authors by extrapolation to infinite salt concentration, are in the range 6.1−10.1% and are therefore appreciably higher than that determined here.

The binding data, cast in the form of a saturation plot of $r$, the ratio of the moles of H1 bound per mole base pairs, as a fraction of $C_f$, the molar concentration of free H1, are shown in Figure 3. In order to determine $K_A$, the intrinsic association constant, and $n$, the saturation value of $r$, the equation for binding at independent sites $r = nK_AC_f(1 + K_AC_f)^{-1}$ (Cantor & Schimmel, 1980) was fitted to the experimental points. It is apparent that the simple binding equation describes quite satisfactorily the results. This analysis yields a $K_A \pm$ standard error of $(3.67 \pm 0.60) \times 10^4$ $M^{-1}$ while $n$ is equal to $(5.55 \pm 0.27) \times 10^{-3}$ or to 1 molecule of H1 bound per $180 \pm 10$ bp. Therefore, the size of the binding site corresponds well to one nucleosome. The occurrence of independent binding can be further verified by plotting $\ln[r/(1 - r)]$ as a function of $C_f$ (lower inset). In

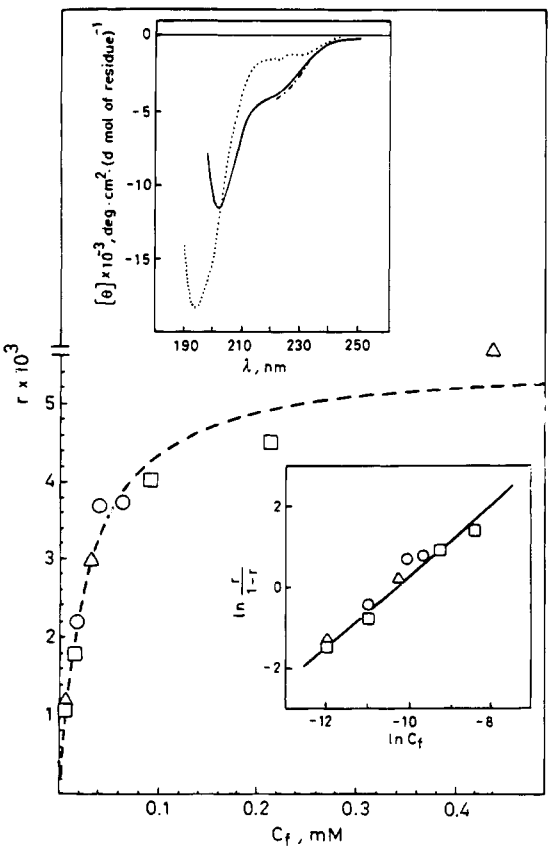


FIGURE 3: Analysis of the binding of H1 to core chromatin. The reconstitution experiments were carried out by slow (□) or fast (○) salt gradient dialysis, as well as by direct mixing (△). The best-fit curve was calculated by the equation for binding at independent sites. The graphical test for independent binding (Hill plot) is shown in the lower inset. The experimental point at $C_f = 0.44$ mM cannot be employed, since it corresponds to a negative value of $r/(1 - r)$. The upper inset shows the circular dichroism spectra of H1 in 20 mM Tris, pH 8, containing 0.6 M KF (—), in DM (— • —) and in 0.01 N HCl (• • •).

such a graph, the slope $\alpha_H$, known as the Hill constant, is an index to the cooperativity (Cantor & Schimmel, 1980). Our data yield a straight line with $\alpha_H = 0.85 \pm 0.06$ over the entire range of $C_f$, which is very close to 1, the theoretical value for noncooperative binding. It must also be noted that in the reconstitution experiments carried out by the method of slow salt gradient dialysis (squares in Figure 3), the counterion concentration was decreased at an average rate as low as 0.025 M per hour in the critical range (0.4−0.15 M) within which reassociation of H1 occurs (Burton et al., 1979) and it is, therefore, reasonable to assume that equilibrium conditions have been approached. The same dependence of $r$ on $C_f$ is, however, obtained by fast dialysis (circles) or direct mixing (triangles), as the corresponding experimental points are distributed randomly around the best line representing the entire set of data. Since chromatin reconstitution by direct mixing at physiological ionic strength does not affect the stoichiometry of binding with respect to the slow reassembly technique, we can infer that under all of the experimental conditions tested here both the histone molecule and the binding site maintain the native configuration involved in specific interactions.

Binding at independent sites has to be expected from a consideration of the molecular architecture of H1 in relation to its location in chromatin. Early digestion experiments of calf thymus nuclei and chromatin with trypsin and micrococcal nuclease suggested that the small, globular domain binds to a cage of three DNA duplexes at the exit point of the chromatosome (Allan et al., 1980). Using a gel mobility-shift assay, Hayes and Wolffe (1993) have recently characterized the binding mode of H1 and H5 to a *Xenopus borealis* somatic 5S RNA gene associated with an octamer of core histones. Their results are in complete agreement with the above model. Before the onset of cooperative interactions, either histone binds preferentially to the 5S DNA wrapped around the histone octamer rather than to naked 5S DNA. As expected, the association of a single linker histone molecule protects 20 bp of free DNA from micrococcal nuclease digestion; interestingly, however, the protected DNA is asymmetrically distributed with respect to the core. The unfolded COOH-terminal domain is believed to be responsible for the neutralization of the phosphate charge, which is required in order to bring the nucleosomes into contact in the higher-order structure (Allan et al., 1986). Since the major structural role of the globular region is to seal the chromatosome, irrespective of chromatin conformation, the involvement of this structural domain in cooperative interactions is quite unlikely. Therefore, the cooperative folding of chromatin into the 30 nm fiber is a necessary condition for the cooperative binding of H1. As we will show below, chromatin condensation is far from being a highly cooperative process.

The results presented by Watanabe (1984) in support of cooperative binding deserve a concise comment. In the reconstitution experiments, this author employed core chromatin obtained from a mixture of core histones and DNA by a stepwise decrease in the ionic strength (Oudet et al., 1975). The characterization of the repeat length of this material was not reported. It has been recognized early (Steinmetz et al., 1978), however, that reassembled core chromatin consists of closely spaced particles in a ~140 bp register, and that the presence of H1 does not appreciably increase this value. The entire matter has been critically examined by Fulmer and Fasman (1979b) and, very recently, advanced by Drew (1993), who has found that only under certain reconstitution conditions (including the use of acetylated core histones and a low salt concentration) the addition of H1 yields a spacing of ~170 bp. All of these observations suggest that in the experiments of Watanabe (1984) binding of H1 occurred in regions of naked DNA interconnecting domains of closely spaced particles. As a fact, above 20−50 mM NaCl, H1 interacts cooperatively with DNA (Clark & Thomas, 1986). Since in this case binding is driven by electrostatic forces, it becomes possible to explain the apparently high value of the association constant ($7.5 \times 10^7$ $M^{-1}$), which indeed is very close to that previously determined by Watanabe and Schwarz (1983) for the binding between protamine and calf thymus DNA ($5.89 \times 10^7$ $M^{-1}$).

*Simple Thermodynamic Model for H1-Induced Folding of Core Chromatin. Energetics and Cooperativity of Condensation.* Having characterized the binding mode of H1 to core chromatin, we can now tackle the intriguing problem of the role of this histone in the stabilization of the higher-order structure, by taking advantage of the capability of DSC to afford directly a measure of the fraction of nucleosomes populating different free energy levels (Balbi et al., 1989; Cavazza et al., 1991), and, therefore, to provide a thermodynamic frame within which to describe the conformational

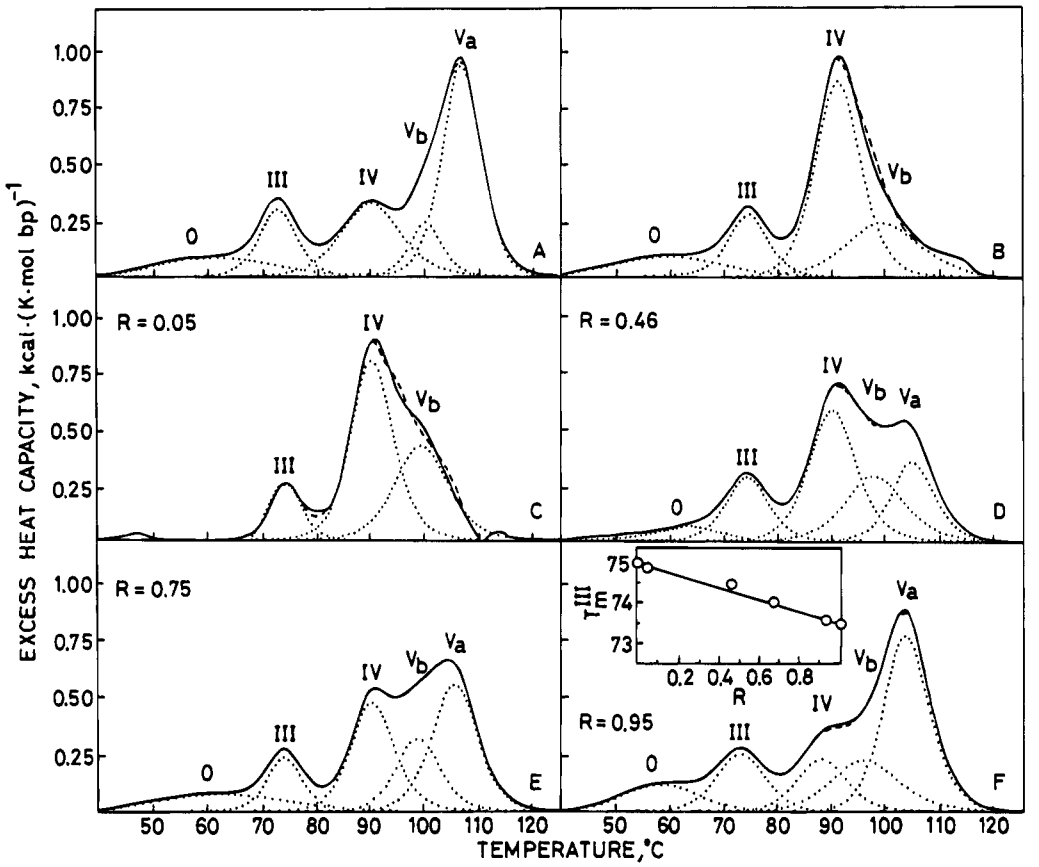


FIGURE 4: Conformational transition of core calf thymus chromatin induced by the progressive binding of H1. (A) Control nuclei; (B) core chromatin; (C−F) reconstituted chromatin. The values of $R$ are indicated. The excess heat capacity curves have been deconvolved into Gaussian transitions. Note that in (C) endotherm 0, corresponding to the unfolding of histones, is erased due to the superimposition of thermal noise. The inset in (F) shows the decrease in the denaturation temperature of the linker induced by the binding of H1.

transitions induced by changes in the external parameters or by binding of ligands. In order to recall briefly the outcome of our previous analysis (Cavazza et al., 1991; Barboro et al., 1993), in Figure 4 we compare the deconvolved excess heat capacity curves of native (A) and H1-depleted (B) nuclei. As shown in a previous section, transition IV at 90 °C, arising from the denaturation of the DNA in noninteracting core particles, dominates the latter thermogram. The concomitant decrease in $\Delta H_m^V$ is further analyzed by considering that transition V can be deconvolved into two subcomponents at 100 and 107 °C, referred to here as $V_b$ and $V_a$, which have been attributed to a "looser" and a "more compact" state of condensed chromatin, respectively (Cavazza et al., 1991; Barboro et al., 1993). The thermogram in panel B clearly shows that while transition $V_a$ disappears when all H1 is removed, endotherm $V_b$ increases with respect to the control (A). Therefore, in core chromatin at physiological ionic strength, some partially ordered state coexists with the fully unfolded one.

Representative excess heat capacity curves, showing the increase in chromatin condensation with increasing $R$, are reported in panels B−F. For $R = 0.95$ (F), the profile of native nuclei is restored. The fractions $f_{IV}$, $f_{Vb}$, and $f_{Va}$ of core particles, populating the states which give rise to endotherms IV, $V_b$, and $V_a$, are obtained by dividing the corresponding transition enthalpies by the total enthalpy change ($\Delta H_m^{IV} + \Delta H_m^{Vb} + \Delta H_m^{Va}$) (Cavazza et al., 1991; Barboro et al., 1993). The values are plotted as a function of $R$ in Figure 5A. Two points emerge from this representation. In the first place, the experimental dependence both of $f_{Va}$ and of $f_{IV}$ on $R$ is sigmoidal; two hyperbolic tangent functions have been fitted to the data. This qualitative result indicates that H1-induced condensation is a cooperative, although not very sharp, transition. In the second place, $\Delta H_m^{Vb}$ decreases monotonically with $R$. A plausible explanation is that the structural change involves two consecutive steps; namely, that the looser chromatin conformation represents an intermediate in condensation. The same conclusion has been drawn from the investigation of the salt-dependent conformational changes of native chromatin, since decondensation at low ionic strength proceeds from $V_a$ to $V_b$ (Cavazza et al., 1991).

Several lines of evidence suggest that the conformation corresponding to transition $V_b$ can be identified with the partially compacted state of core chromatin characterized by Yao et al. (1991) and Garcia-Ramirez et al. (1992) using hydrodynamic methods and electron microscopy. H1-depleted oligonucleosomes fold by rising the monovalent salt concentration up to 0.1−0.2 M; the folded structure corresponds to a zig-zag array of consecutively contacting nucleosomes, and can also transiently condense into a solenoid (Hansen & Ausio, 1992). Intercore interactions act as the driving force for bringing consecutive nucleosomes into contact and bending the linker DNA. It is important to note that when all these observations are taken together, the picture emerges that in the course of both the salt- and H1-induced condensation the higher-order structure nucleates from a partially ordered state rather than from the unfolded polynucleosomal chain. This represents, of course, a central point for any dynamic model for chromatin folding.

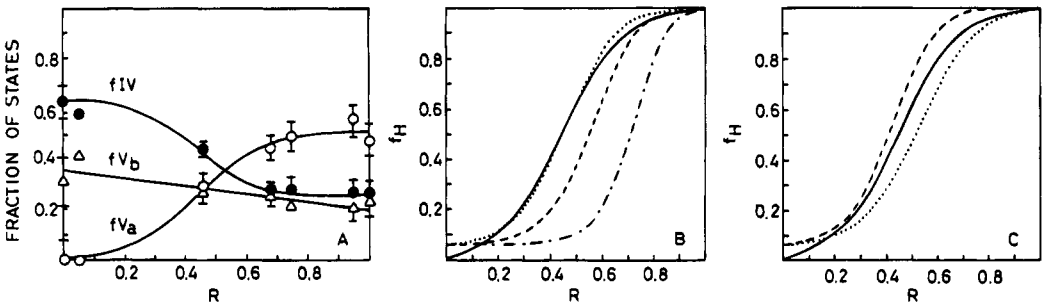


FIGURE 5: (A) Experimental dependence of the fraction of core particles which denature in transitions IV, $V_b$, and $V_a$ on $R$. The points represent the mean ± standard deviation of from three to five determinations. (B, C) Comparison of the experimental (—) and theoretical transition curves calculated for different values of $\Delta G^{int}$ and $c$. (B) $\Delta G_0^c$, $\Delta G^{int}$, 1.5 and −5 kcal per nucleosome mole, respectively; $c$ = 2 (•••), $c$ = 3 (- - -), $c$ = 6 (— • —). (C) $c$ = 2, $\Delta G_0^c$ = 1.5; $\Delta G^{int}$ = −4 (•••) and −6 (- - -) kcal per nucleosome mole, respectively.

Before we present a simple thermodynamic treatment of chromatin condensation, a few basic features of the DSC profile relating to the status of chromatin in cells from different sources must be considered. In resting cells (calf thymocytes and rat hepatocytes), the average degree of condensation, as determined by the expression $\Delta H_m^V/(\Delta H_m^{IV} + \Delta H_m^V)$, is about 0.7 (Barboro et al., 1993; and this work). This value decreases in actively dividing cells, and is close to zero in transformed rat hepatocytes of preneoplastic persistent nodules (Barboro et al., 1993). This dependence of the melting behavior on the functional state must be taken into account in the interpretation of the reconstitution experiments. When all H1 is removed, transition $V_a$ disappears; upon binding of H1, the melting profile of native nuclei is regained. Even when reconstitution was carried out in the presence of a large excess of H1, we were unable to detect any appreciable increase in the degree of condensation with respect to the control. This observation indicates that a fraction of the genome is permanently unfolded, independent of H1 binding, and we have hypothesized that it comprises the set of genes competent for transcription in a differentiated cell (Barboro et al., 1993). Our model, of course, applies only to the main fraction of bulk chromatin which undergoes reversible condensation with H1 binding. Therefore, for the purpose of comparison with the experimental results, the dependence of $f_{Va}$ on $R$ (Figure 5A) has been converted to $f_H$, the fraction of core particles which exist in the condensed state, defined by the ratio between the value of $f_{Va}$ for a given $R$ and the one determined at saturation ($R$ = 1). The plot of $f_H$ against $R$ is shown in Figure 5B.

In our simple model, the Gibbs free energy of condensation (or folding) of chromatin per nucleosome mole, $\Delta G^c$, is defined with respect to $\Delta G_0^c$, the free energy of formation of the condensed state in the absence of H1 (namely, for core chromatin). If $\Delta G_b^u$ and $\Delta G_b^f$ are the free energies of combination of H1 with the unfolded and folded core chromatin, respectively, we can write

$$\Delta G^c = \Delta G_0^c + \Delta G_b^f - \Delta G_b^u + \Phi(R)\Delta G^{int} \quad (1)$$

where $\Delta G^{int}$ is the average free energy change per nucleosome when H1-bound nucleosomes interact in the folded conformation and $\Phi(R)$ is the fraction of interacting nucleosomes. The free energy of combination is given by the equation (Schellman, 1955):

$$\Delta G_b = -RT \ln(1 + K_A[H1]) \quad (2)$$

As we have shown previously, the binding constant $K_A$ does not dependent on chromatin conformation, so that $\Delta G_b^f$ and $\Delta G_b^u$ cancel out in eq 1, and the fraction of nucleosomes in the helical state is given by

$$f_H = 1 - \{1 + \exp[-(\Delta G_0^c + \Phi(R)\Delta G^{int})/RT]\}^{-1} \quad (3)$$

The model is expected to take into account adequately accepted molecular aspects of H1-induced condensation, as the globular domain recognizes the specific binding site at the exit points of the chromatosome, irrespective of the conformation, while helix-stabilizing interactions among nucleosomes arise from the shielding of the phosphate charges by the COOH-terminal domain.

In order to evaluate $\Phi(R)$, the chromatin loop is taken to be a linear array of $N$ binding sites placed in fixed distances, each site corresponding to a nucleosome; the chain is very long, so that end effects can be neglected. Since binding of H1 occurs randomly, the probability of each site being occupied is $R$. H1-bound nucleosomes will form clusters containing 1, 2, ..., $k$ elements. The total number of clusters of size $k$ ($n_k$) is given by (Stauffer, 1985)

$$n_k = NR^k(1 - R)^2 \quad (4)$$

Above a critical value of the size, the cluster will be able to condense in a stable helical conformation. Therefore, $H$, the total number of sites which contribute to the stability of the helical state for a given $R$, is

$$H = N\sum_{k \geq c}^{N} kR^k(1 - R)^2 \quad (5)$$

where $c$ is the critical size corresponding to the onset of stabilizing interactions. By dividing eq 5 by $N$, we obtain the normalized fraction $\Phi(R)$ of interacting nucleosomes, which is the result we sought.

In Figure 5B, the experimental dependence of $f_h$ on $R$ is compared with the theoretical curves (eq 3) calculated for different values of $\Delta G_0^c$, $\Delta G^{int}$, and $\Phi(R)$. It can be seen that the data are satisfactorily predicted using $\Delta G_0^c$ and $\Delta G^{int}$ = 1.5 and −5 kcal per nucleosome mole, respectively, and by evaluating the sum in eq 4 for $c$ = 2. The result is extremely sensitive to the choice of the critical value of $k$, since the curves obtained for $c$ = 3 and 6 progressively shift to higher values of $R$, and tend to become asymmetrical; for $c$ = 6, $f_h$ increases slowly up to $R$ = 0.5 and quite abruptly above. The sensitivity to the value of $\Delta G^{int}$ is also shown

for $c = 2$; appreciable deviations from the experimental trend are observed for −4 and −6 kcal per nucleosome mole, respectively. At present, we cannot attach a precise molecular meaning to the value of $\Delta G^{int}$. The binding both of the $NH_2$-terminal tails of core histones (Libertini et al., 1988) and of H1 to the linker (Yao et al., 1991) may be involved in the modulation of the attractive forces among nucleosomes. This complex scenario is complicated further on by the free energy of bending of the linker, which must be taken into account in the thermodynamic analysis of the tight association of consecutive nucleosomes (Yao et al., 1990).

The fundamental question to ask is whether condensation is a cooperative process. The answer is yes, as it can be verified using very simple arguments. The experimental transition curve (Figure 5B) shows that the fraction of nucleosomes in the condensed state is lower than $R$ below $R = 0.4$, but higher above. This, of course, qualitatively shows that beyond a critical value of the fraction of reconstituted nucleosomes small neighboring clusters merge, by incorporating in the helical turns H1-depleted nucleosomes. On the other hand, our experiments definitely rule out that condensation can be described in terms of a two-state or "all or none" transition between the unfolded state and the 30 nm fiber. In order to form the first helical turn in the solenoidal model (Widom & Klug, 1985), six nucleosomes must be "frozen" before a favorable (edge to edge) interaction can be established between nucleosome $i$ and nucleosome $i+6$. The model predicts that also the faces of consecutive nucleosomes interact, although their radial array prevents the stacking from being very effective. Clearly, if the transition would proceed directly from the core to the 30 nm fiber, condensation should be observed only in the limit $R = 1$. Since comparison of the model with the experiments shows that complete agreement is observed using $c = 2$ in the computation of $\Phi(R)$, we infer that clusters consisting of few nucleosomes exist in some helical array stabilized by the same basic interactions (face to face and edge to edge) which prevail in the fully folded 30 nm fiber. In conclusion, our results confirm that the growth of the helix involves several intermediate states. The continuous folding of the polynucleosomal chain, associated with the sharpening of the bands at 57 and 110 Å in the low-angle X-ray patterns, has been described in a previous physicochemical study of the salt-induced condensation (Widom, 1986). As far as structural aspects are concerned, an early model for the progressive compaction of H1-containing chromatin into helices with increasing numbers of nucleosomes per turn, built on exhaustive electron microscope observation (Thoma et al., 1979), deserves a careful reexamination. In our laboratory, we are now attempting to establish, by an improved DSC technique, whether the 107 °C endotherm can be used not only to measure the fraction of condensed nucleosomes but also to detect slight variations in the compaction of the ordered state.

Not only the growth but also the nucleation of the helical state is poorly understood at the molecular level. Assuming that the first nucleation event involves the conversion of a partially folded structure [for example, the zig-zag of contacting nucleosomes described by Garcia-Ramirez et al. (1992)] into a solenoid with a low number of nucleosomes per turn (Hansen & Ausio, 1992), the mechanism by which an edge to edge array is converted into the radial disposition around the solenoid axis remains to be explained. It is legitimate to hypothesize that a conformational change of the linker, induced by interaction with the COOH-terminal domain of H1, affects this transition; on the other hand, bending of the linker in oligonucleosomes occurs whenever the chain folds, independently of the presence of the histone (Yao et al., 1991). However, the status of the linker is appreciably affected by H1 binding *per se*. As shown in the inset in Figure 4F, $T_m^{III}$, the melting temperature of this domain, decreases by about 1.5 °C in the course of reassociation. This small but significant effect is comparable with the depression in $T_m^{III}$ which occurs in the course of the salt-induced refolding of native nuclei (Cavazza et al., 1991). Therefore, besides the suppression of repulsive electrostatic forces, H1 could play an additional role in determining the correct spatial positioning of nucleosomes in the early stages of condensation.

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