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NATIVE CHROMATIN–DNA STRUCTURE AND CELL CYCLE: DIFFERENTIAL SCANNING CALORIMETRY AND GEL ELECTROPHORESIS

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ABSTRACT

Differential scanning calorimetry (DSC) and gel electrophoresis have been carried out on intact nuclei and on the corresponding chromatin extracted, using the two most common procedures, from rat hepatocytes, as a function of shearing, ionic strength and DNA-ase digestion.

These studies, along with those obtained on free DNA fragments of widely different length, suggest that the chromatin prepared by limited nuclease digestion lacks the topological constraints present in situ, which appear instead to be preserved even at low ionic strength (0.01 M) by lysis of the native nuclei in cold hypotonic buffer and by subsequent gentle resuspension (without shearing) of the viscous chromatin mass.

While both unsheared chromatin preparations contain a similar repeating structure, only the chromatin prepared by limited nuclease digestion is lacking the higher molecular-weight bands present in native nuclei. Furthermore only "cold water" chromatin reveals significant cell-cycle-related thermodynamic alterations which exactly mimic those apparent in situ from the heat capacity profiles of corresponding nuclei.

Results are discussed in terms of DNA packing/supercoil in vivo which could modulate during cell-cycle progression and which could be maintained by the topological constraints existing along native nucleofilaments.

INTRODUCTION

The genome of eukaryotes is organised into chromatin subunits known as nucleosomes, which form the basis for its higher order folding in the nucleus (for a review, see refs. 1 and 2). Although a great deal of structural information has been accumulated on interphase chromosomes at the level of the polynucleosomal organisation, known as the 300 Å thick fibre [3–6], and at higher levels [1,2,5,7–10], considerable controversy still exists concerning the details of the higher order folding for the truely native chromatin structure, despite the significant progress made possible by low-angle X-ray

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diffraction studies [7,8] and high resolution image analysis [1,10,11] on metaphase chromosomes, intact nuclei and cells.

Since its early discovery more than 10 years ago [12,13], studies on the 300 Å thick fibre may be divided into two major groups, the larger using homogeneous solutions of chromatin prepared by limited nuclease digestion [14] and the other using the viscous mass of native chromatin brought gently into suspension without shearing [15]. Shearing, typically carried out in a motor-driven homogeniser to obtain the perfectly homogeneous solution needed for most early chromatin studies, has been shown to cause the irreversible disappearence of the 300 Å thick fibre in both chromatin preparations [14,15].

However, large and irreconcilable differences remain among the two chromatin preparations which may have profound bearing on the significance of structural and functional studies being carried out on chromatin prepared by limited nuclease digestion. Indeed, at low ionic strength the latter displays a lack of 300 Å thick fibres or of polynucleosomal quaternary DNA structure, which are present in the former [2,4,5,12,16]. Furthermore, in the latter the fibre appears as a solenoid at high ionic strength [3,6,13] rather than as a multifilament rope [5,17].

These differences can be resolved by characterising the two common chromatin preparations in a variety of conditions by means of two non-invasive biophysical probes, namely circular intensity differential scattering [18–20] and differential scanning calorimetry (DSC) [16,21,22]. Only now are these techniques capable of characterising large biopolymers like chromatin, both in vivo and in vitro, with a high degree of resolution.

This paper uses DSC to monitor the heat exchanged by the macromolecule under study versus temperature; the acquired thermodynamic parameter (enthalpy) is related to the composition and structural alterations of the examined biopolymer, allowing information to be obtained in a temperature range unreachable by traditional spectroscopic methods (above the boiling point of water) but uniquely informative about the well-known increase in thermal stability of the higher order structure of biopolymers at physiological ionic strength [16,21,22].

Measurements performed in a temperature range between 320 K (47 °C) and 410 K (137 °C) on intact cells, nuclei and isolated chromatin from rat liver provide thermal capacity profiles whose peaks are known to be related to the denaturation of various macromolecules (proteins, chromatin, DNA or RNA) and particularly to the higher order DNA structures [16,21,22].

The DSC experiments described in this paper, despite their inherent limitations, if correlated with gel electrophoresis either in agarose or polyacrylamide, give useful information about the native chromatin–DNA structure and about its changes occurring in situ during cell cycle progression. The results reported here, along with those obtained on free DNA fragments of widely different length, do not appear to contradict recent or earlier

observations obtained from a wide range of biophysical probes [7,8,9,11,16,17,21] and suggest that structurally important topological constraints existing in vivo are lost in chromatin prepared by limited nuclease digestion.

MATERIALS AND METHODS

Cell and nuclei isolation

Sprague Dawley male rats of 300 g body weight were anaesthetised with 50 mg kg^{-1} of penthotal injected i.p. Cells and nuclei were isolated from the perfused liver following the usual procedure [12]. All procedures were carried out at 4° C.

Chromatin isolation

As previously described [15], the nuclear pellet was washed with 10 volumes of 0.15 M NaCl and 0.01 M Tris–HCl, pH 8, and allowed to swell in cold (4° C) distilled water for 60 min in an ice bath. Nuclei were lysed by gentle homogenisation and chromatin was purified by centrifugation through 1.7 M sucrose at 100 000 × g for 80 min. This chromatin was gently, by 20 strokes in a loose Dounce homogeniser, resuspended either in low (0.01 M Tris–HCl, pH 8) or physiological (0.15 M NaCl, 0.01 M Tris–HCl, pH 8) ionic strength buffer, and centrifuged for 15 min at 4000 × g in order to obtain a concentrated sample ready for calorimetry. We refer to this as the "unsheared" chromatin prepared by the "cold water" method. Occasionally, to monitor the effect of mild shearing, chromatin was "vigorously" brought into suspension by more than 50 strokes in the loose Dounce and also by using the insulin syringe.

Extensive shearing was accomplished by homogenizing for 90 s either in a Sorvall vortex homogenizer at full speed or in an ultrasonic sonicator at 50 Watt.

Chromatin was also extracted from nuclei by the most common methods of limited micrococcal nuclease digestion as described by Noll et al. [14]. Samples of nuclei were suspended in 0.34 M sucrose buffer A (suspension was made 1 mM in $CaCl_2$) and digested with 10–15 U ml^{-1} micrococcal nuclease (Sigma) for different periods at 37° C (30–45 s). The reaction was stopped by addition of EDTA to a final concentration of 10 mM.

Nuclei were centrifuged for 5 min at 4000 × g, lysed (as above) and centrifuged for 2 min at 4000 × g: the supernatant contains chromatin soluble at all ionic strengths, while the pellet [4,23] contains chromatin insoluble at low ionic strength (0.01 M). Alternatively chromatin was extracted from the nuclei by digestion with 50 U ml^{-1} of micrococcal nuclease for 7 min.

DNA isolation

High molecular weight DNA was isolated from rat liver hepatocytes. The cell pellet at a concentration of 10^8 cells ml^{-1}, was resuspended in 10 vol of 0.5 M EDTA, 0.5% SDS and 100 $\mu g\ ml^{-1}$ proteinase K and was placed in a 50 °C water bath for 3 h. The DNA is extracted with phenol–chloroform–isoamylic alcohol to yield a final DNA precipitate with ethanol, as described by Maniatis et al. [24].

Agarose gel electrophoresis

Chromatin was incubated at 4 °C overnight in 8 M urea, 2 M NaCl, 1% SDS and 0.01 M Na_2EDTA to detach the histone proteins from DNA; the DNA was then extracted as above.

The DNA extracted was analysed by an electrophoretic run in either 0.4 % (see later, Fig. 10) or 0.8% agarose (see later, Figs. 4 and 12) at 100 V (60 mA) for 6 h. The resulting patterns visualized by irradiation at 302 nm, after ethidium bromide staining (to a final concentration of 1 $\mu g\ ml^{-1}$) were photographed with a Polaroid or a Reflex camera provided with a Wratten filter 9. The photographic negatives were scanned densitometrically.

Alternatively, the patterns were computer-acquired via a TV camera, digitally enhanced with proper filtering, and displayed. The acquisition was performed using a $512 \times 512 \times 8$-bits frame-grabber board working at 30 frames s^{-1} and the final image was obtained after averaging over several pictures.

Polyacrylamide gel electrophoresis

Enzymatically prepared chromatin was separated by electrophoresis using 2.5% acrylamide/0.5% agarose (acrylamide: *N,N'*-methylenebisacrylamide, 20 : 1) with TBE (Tris 0.05 M, boric acid 0.05 M, Na_2EDTA 1 mM) as the buffer system [25]. Samples were loaded in 10% sucrose, 2 mM EDTA and 0.25% Bromophenol blue. The run was 5 h at 150 V (80 mA). Ethidium bromide staining was performed by layering ethidium bromide solution (2 $\mu g\ ml^{-1}$) on the gel for 1 h and washing excess stain from the gel with $MgSO_4$ solution (1 mM).

Analytical procedures

DNA concentration of chromatin was determined by UV absorption at 260 nm in 0.2% SDS using an extinction coefficient of 21 000 $cm^3\ g^{-1}$ [15], or by the method of Munro and Fleck [26]. Protein concentration was determined by the method of Hartree, employing bovine serum albumin as a standard [27].

Differential scanning calorimetry

DSC experiments were performed on a Perkin–Elmer DSC-2C with 75 μl aluminium capsules, in a temperature range between 320 and 410 K.

In order to increase the reproducibility and sensitivity of measurements, instead of a standard data recovery signal, such as a chart recorder, an electronically interfaced computerized system was used offering great possibilities of acquisition, background subtraction and display of calorimetric data. With this computerized system, it is possible to attain high performance filtering at both hardware and software levels [22,28].

In order to record even very weak signals, scanning was performed at a low rate (5 °C min^{-1}), with high sensitivity (0.1 mcal s^{-1}) and high sample size (about 60 mg $capsule^{-1}$).

RESULTS

The capacity profiles analysed in this section were extracted from properly processed raw calorimetric data [28]. In Fig. 1 the raw data, obtained from G0 nuclei, are illustrated in order to show both the reproducibility of the measurements and the first step in data processing. A further data enhancement process has been performed [28] in order to obtain a better peak identification (Fig. 2A).

Confirming recent data [22], differential scanning calorimetry of native G0 nuclei (Fig. 2A), performed between 320 and 410 K, reveals on average four thermal transitions, with those at 365 and 375 K being dominant. Progressive digestion of G0 nuclei with 500 U ml^{-1} of DNA-ase [29] reveals only a selective reduction of the transition at 328 K at an early time (7 min); whereas at a later time (90 min), the melting enthalpy at 365 K becomes dominant at the expense of a complete disappearance of the transition at 375 K (Fig. 2B, C).

A similar effect is present in the heat capacity profiles of the same native G0 nuclei following the limited nuclease digestion which is used in the most conventional procedure for chromatin isolation, typically 15 U ml^{-1} of micrococcal nuclease for 30 s (Fig. 2D). More extensive digestion (not shown) of nuclei with 50 U ml^{-1} of micrococcal nuclease for 7 min causes further reduction in the average size of chromatin–DNA fragments but yields a similar thermal profile, consistently displaying the disappearance of both the transitions at 328 and 375 K present in undigested nuclei (Fig. 2A). In both cases, a fifth (mostly hidden) transition in the range 335–355 K becomes evident.

After the in situ interphase chromosomes are first cut by the nuclease into fragments smaller than those present in the undigested nuclei (as shown by

the agarose gel electrophoresis of Fig. 3), the nuclei are gently lysed in hypotonic buffer to give the well-known soluble chromatin [14].

At low ionic strength, similar thermal profiles are obtained for both the soluble and insoluble (at 0.01 M) chromatin subfractions, with only the two intermediate transitions being present (Fig. 4A); this appears to be true following either limited (10 U ml^{-1} for 30 s) or extensive (50 U ml^{-1} for 7 min) digestion. The transition occurring at 345 K (shown to correspond to melting of the protein [17]) is more pronounced in the insoluble fraction (not shown).

The well-known decrease in the average molecular weight of DNA fragments (Fig. 3) also appears to be associated with a decrease in the melting temperature of the last transition, from 365 K in undigested nuclei to 360 K in the mildly digested nuclei.

The mean values and standard deviations of the melting enthalpies of the various transitions are summarized in Table 1.

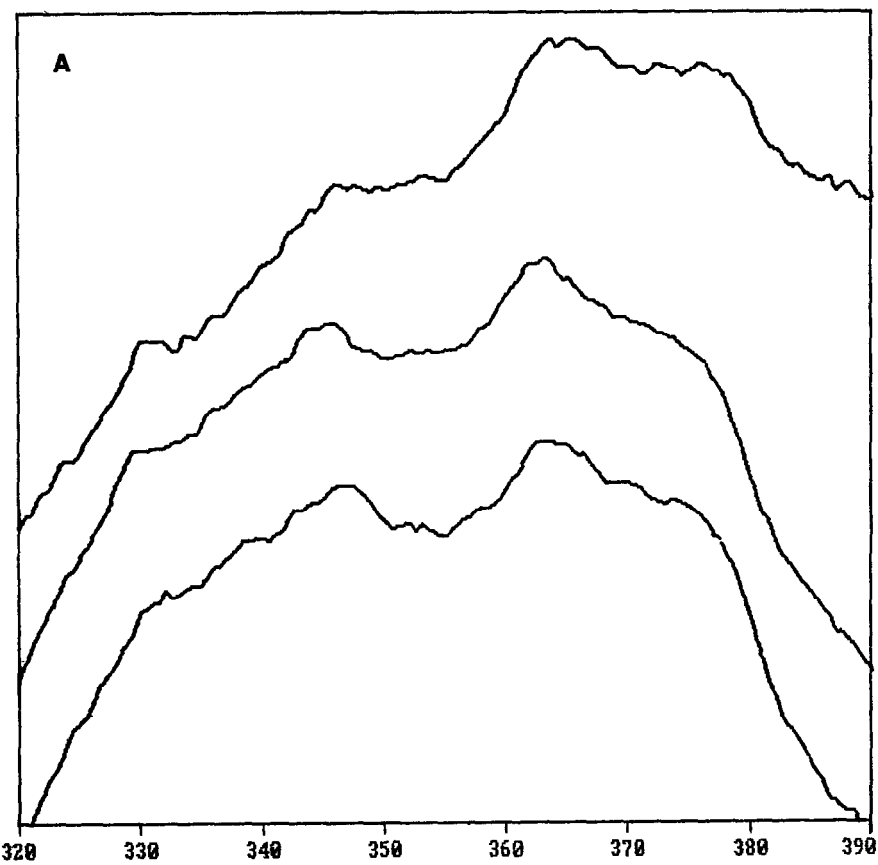

Fig. 1. Heat capacity profiles of different preparations of G0 nuclei undigested, before (A) and after (B) background subtraction.

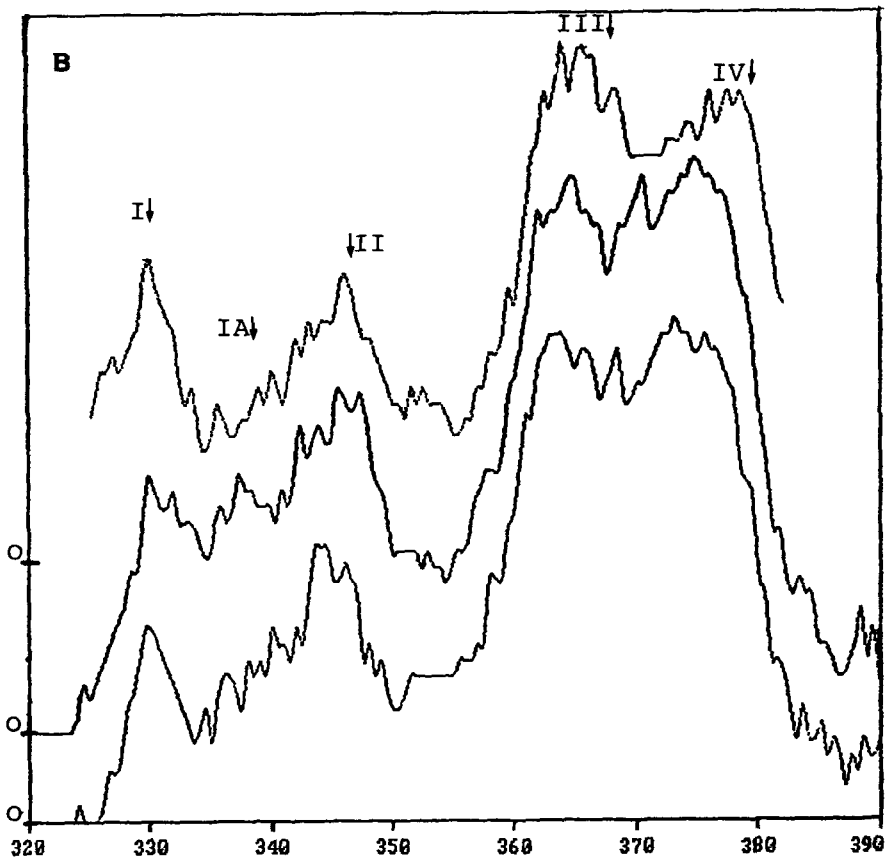

Fig. 1 (continued).

TABLE 1

Melting enthalpies and transition temperatures (K) for G0 nuclei as a function of DNA-ase and nuclease digestion. Melting enthalpies are expressed in terms of calories per gram of nuclear DNA pellet

Sample	Transition				
	I	IA	II	III	IV
Undigested	2.9 (325–335)	–	7.2 (340–352)	9.7 (358–370)	12.1 (371–385)
DNA-ase digested 500 U ml^{-1}		1.28	3.23	10.4	11.78
7 minutes	–	(330–340)	(340–352)	(358–370)	(371–385)
DNA-ase digested 500 U ml^{-1}		0.2	1.3	8.5	–
90 minutes		(330–340)	(341–352)	(358–370)	
Nuclease-digested 10 U ml^{-1}	–	0.8	1.4	4.9	–
45 seconds		(330–340)	(341–352)	(354–368)	

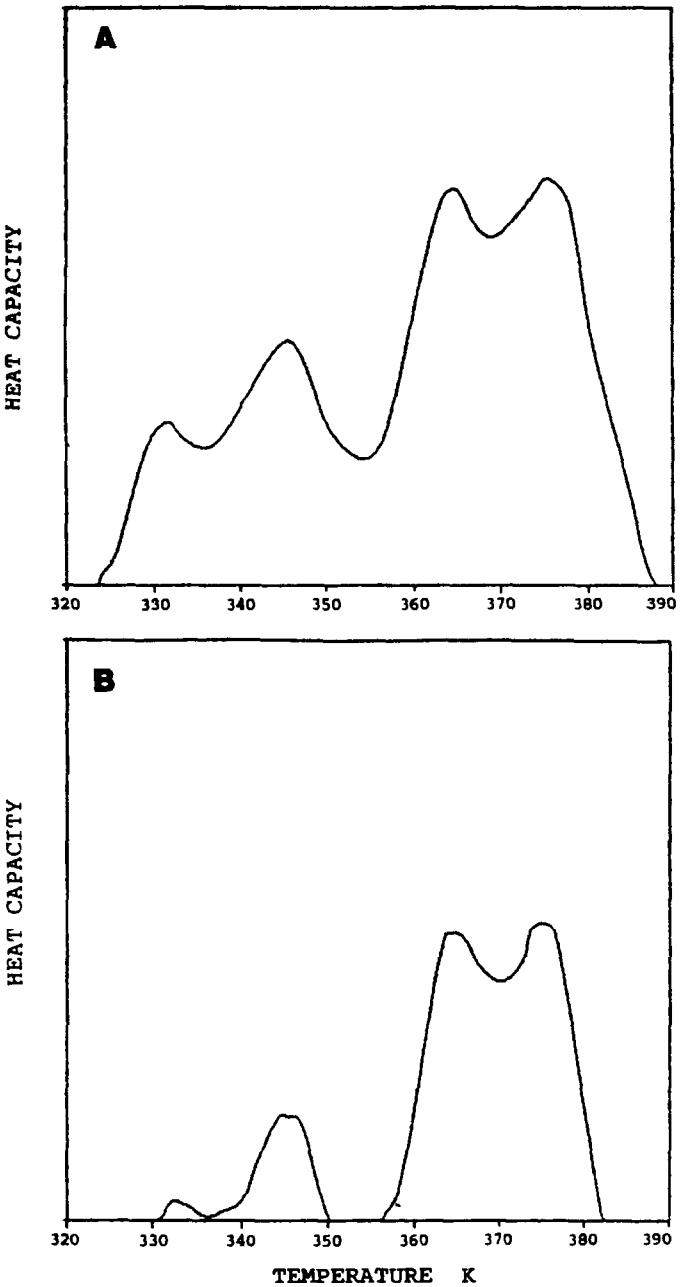

Fig. 2. Effect of enzyme digestion on the heat capacity profiles of G0 nuclei: undigested (A), DNA-ase (500 U ml^{-1}) digested for 7 min (B), and for 90 min (C), and micrococcal nuclease digested for 30 s at 15 U ml^{-1} (D). The raw data are acquired on-line and smoothed with a minicomputer, to be then subtracted from the background (dotted lines) for melting enthalpy determination. The effect of our routines being interfaced with an AT-IBM personal computer is shown in Figs. 1 and 2.

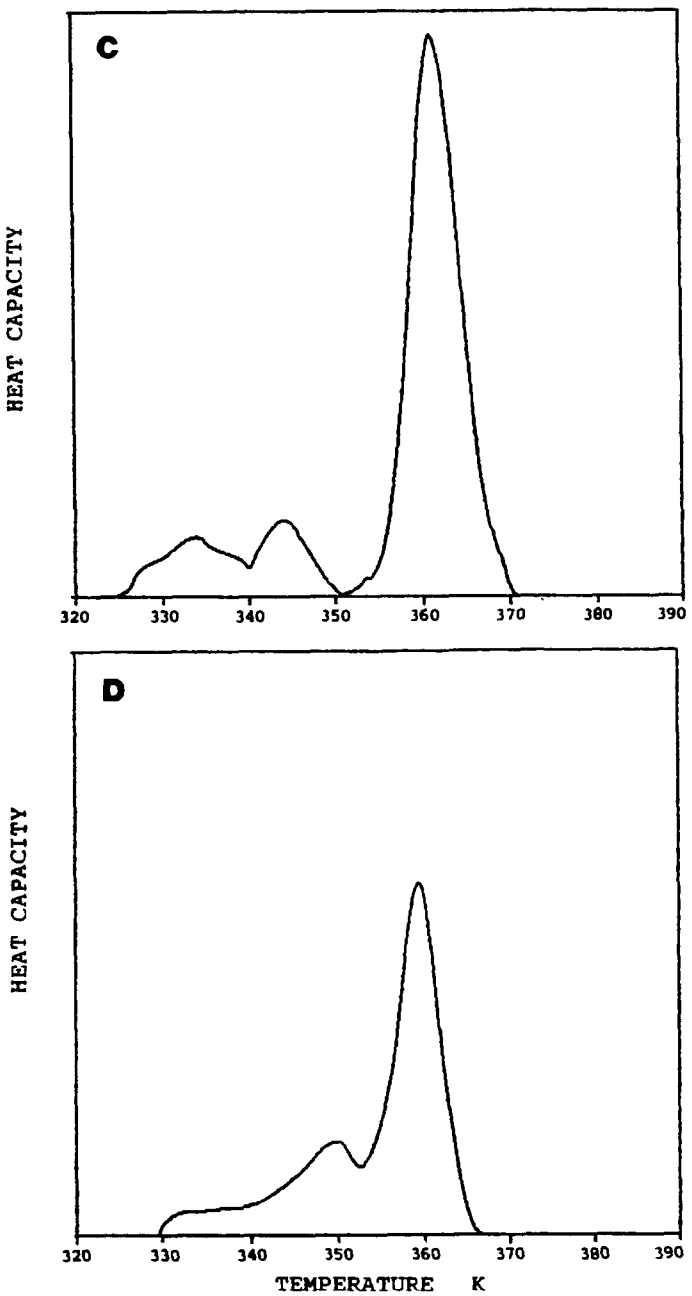

Fig. 2 (continued).

The heat capacity profiles of the viscous chromatin pellet (Fig. 6), sedimented from G0 native nuclei immediately after lysis in cold water without the use of any nuclease, and gently resuspended with a few strokes of a loose Dounce homogeniser [17], indicate, at every ionic strength, the last

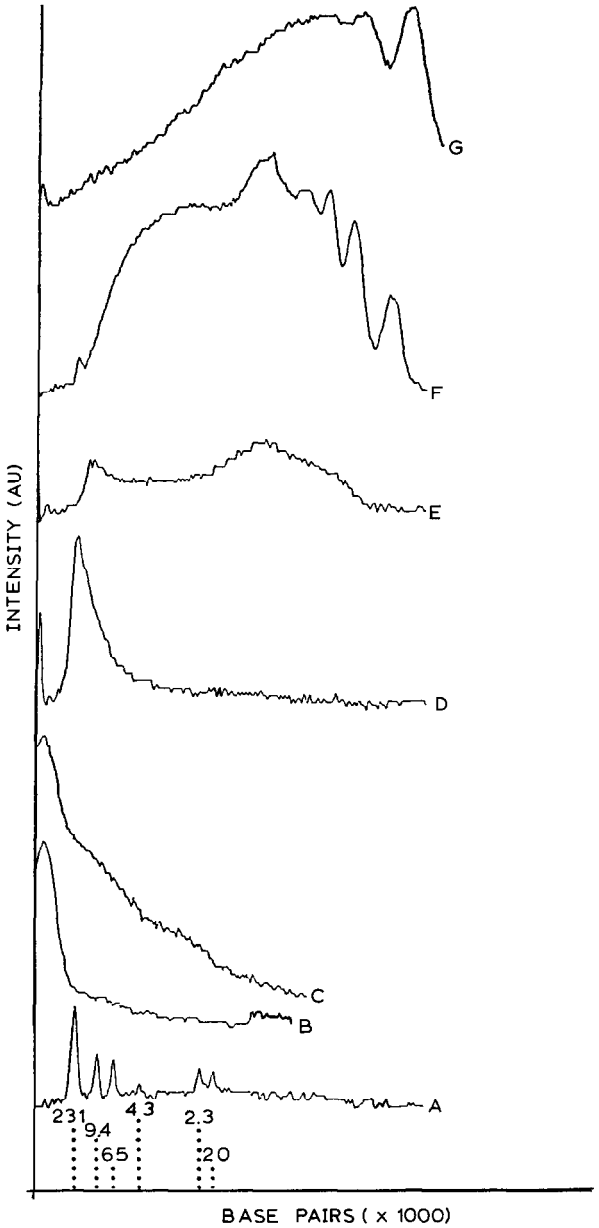

Fig. 3. Size determination of DNA extracted from various G0 chromatin preparations by analysis on 0.8% agarose gel as described in the text. From the bottom, λ-DNA markers (A); chromatin prepared by cold water method and unsheared (B), brought into suspension gently (C), vigorously (D), and brought into homogeneous solution by ultrasonication (E); chromatin prepared by digestion with nuclease 15 U ml^{-1} for 45 s, before (F), and after (G), redigestion with 20 U ml^{-1} for 45 s. The digitized normalized densitometric profiles are shown.

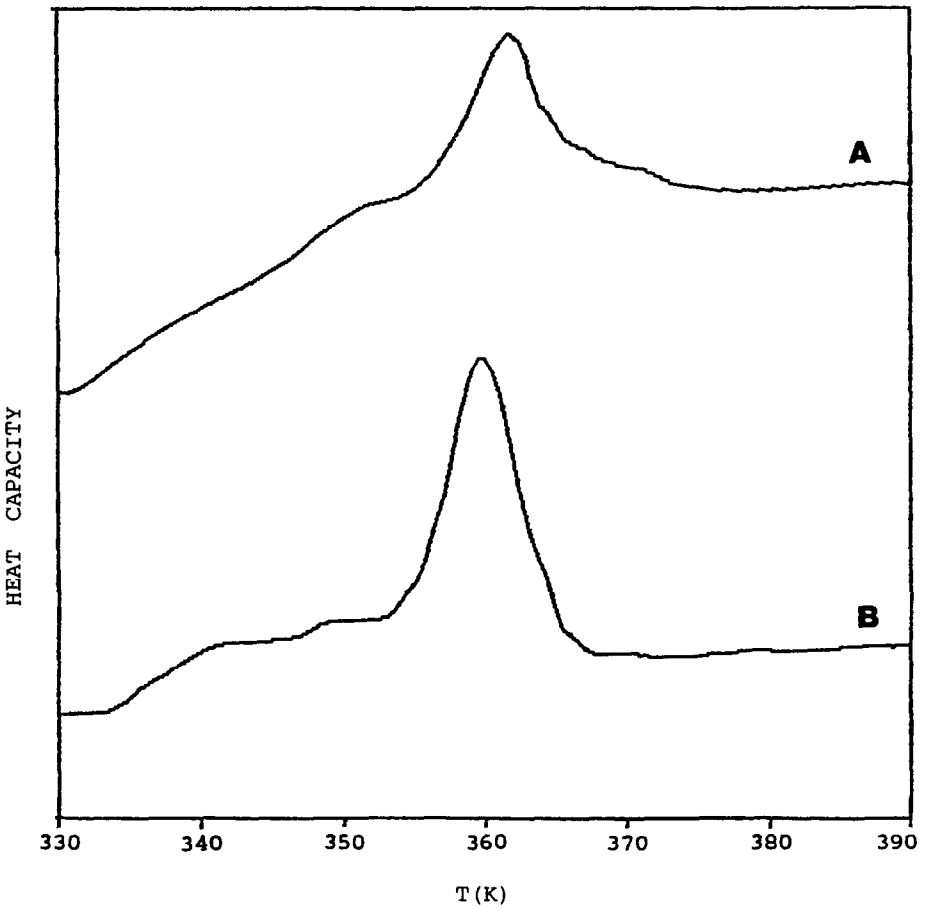

Fig. 4. Heat capacity profiles of soluble chromatin prepared by the nuclease method at 10 mM Tris–HCl pH 7 (A), and at 150 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris–HCl pH 7 (B). Digestion is carried out with 10 U ml^{-1} for 30 s.

three transitions present in native G0 nuclei with only a slight shift towards lower temperatures (see also refs. 16 and 21). This is referred to as the "cold water" method. At low ionic strength, the fourth transition at 372 K is consistently apparent in unsheared chromatin, even if the isolation procedure,i.e. the number of strokes to bring it into suspension, appears critical in determining both the size of the chromatin–DNA fragments and the enthalpy change (in cal g^{-1} and in percentage) associated with this fourth transition (see Table 2). Only when the isolated chromatin is also vigorously passed through an insulin syringe to decrease the DNA molecular weight to about 23 Kbp, (Fig. 3), does the thermal transition at 372 K disappear at low ionic strength. However, it is reassuring that, in all cases at higher ionic strength, the percentage of "cold water" chromatin melting at 375 K

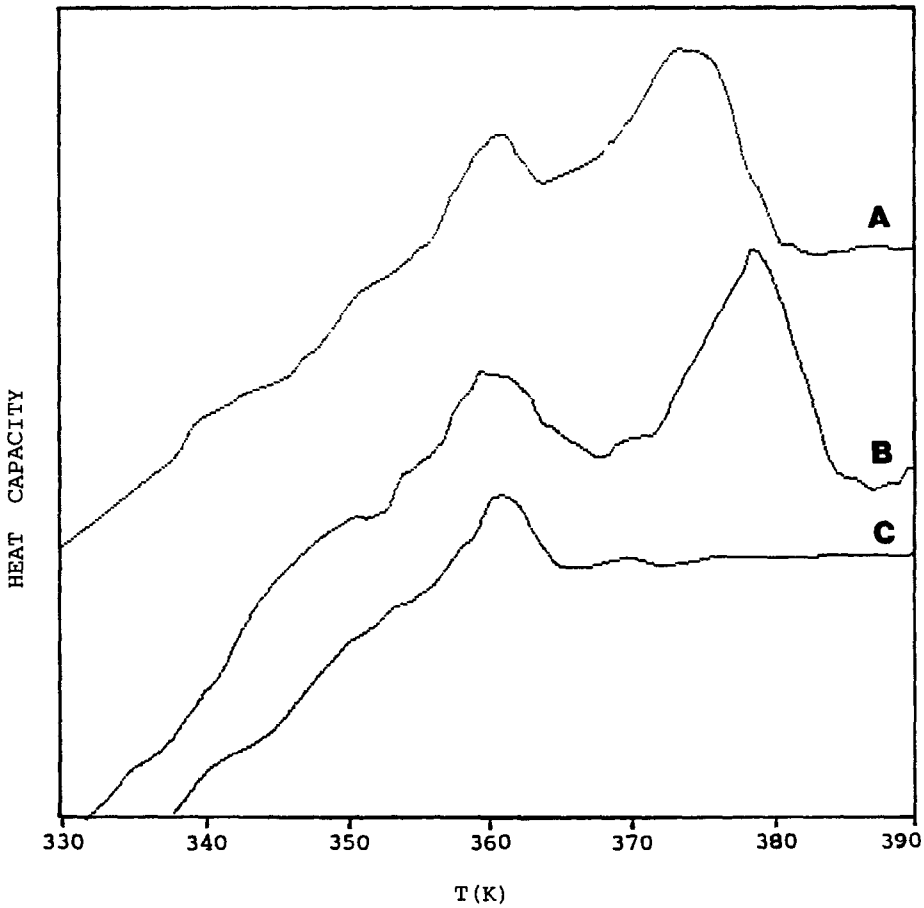

Fig. 5. Heat capacity profiles versus temperature of G0 chromatin prepared by the cold water method, unsheared at low (A) and high (B) ionic strength, or sheared at low ionic strength (C). The molecular weight of these preparations can be seen in Fig. 2, D and E profiles respectively.

consistently rises towards the level of the intact nuclei, even with vigorous resuspension (Fig. 5).

Only with extensive mechanical shearing, which homogenises chromatin into a broad smear of small DNA fragments with an average size of less than 2 Kbp (Fig. 3 and references 13–17), is the fourth transition consistently absent, even at the physiological ionic strength of 150 mM NaCl, 10 mM Tris–HCl (pH 8) and 5 mM $MgCl_2$ (Fig. 6); at the same time, the melting enthalpy of transition III becomes only 4.0 cal g^{-1} (Table 2).

At the same physiological ionic strength, the chromatin prepared by limited nuclease digestion shows a similar profile, but lacks transition IV at 375 K (Fig. 4B) and displays DNA fragments smaller than 20 Kbp. These have an average size of about 6 Kbp for the milder micrococcal digestion (10

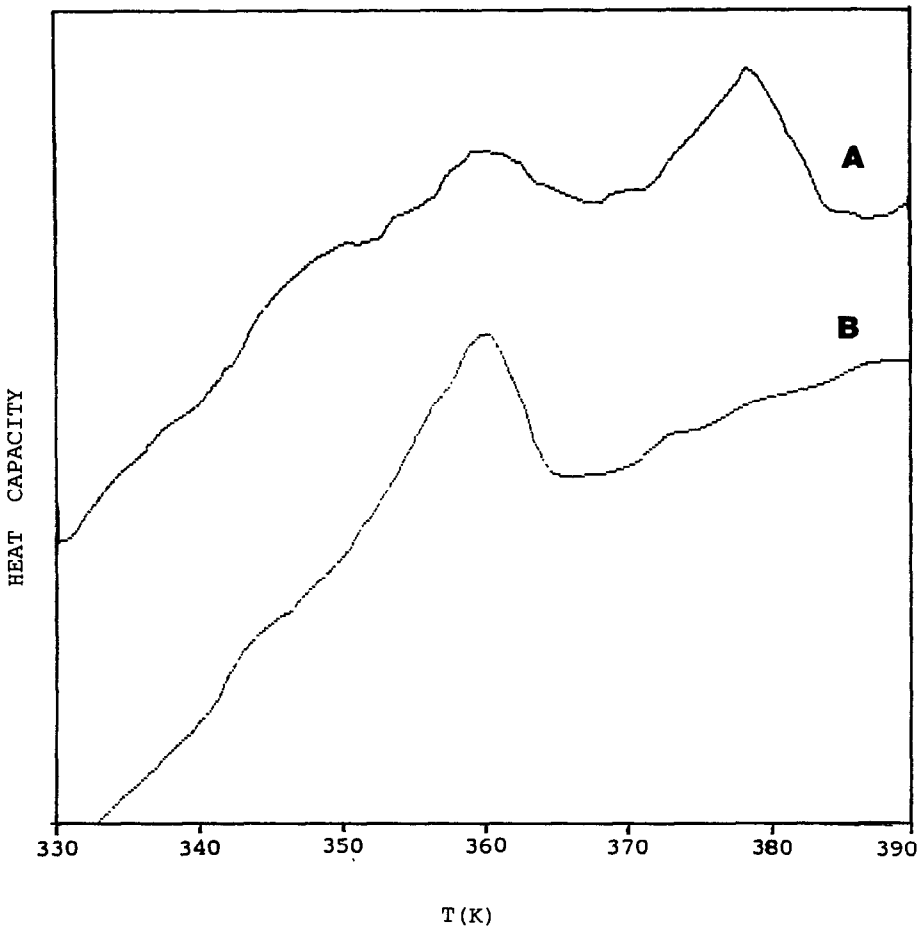

Fig. 6. As Fig. 3, but for the viscous chromatin gel obtained by the "cold water" method at physiological ionic strength, before (A) and after (B) shearing with ultrasound at 50 watt for 90 s.

U ml^{-1} for 30 s) (Fig. 3). The melting enthalpy of transition III at 360 K is about 9.2 cal g^{-1}, which is identical to the in situ value (Table 1), whereas the corresponding value at low ionic strength is only 4.9 cal g^{-1} at 365 K (Table 2 and Fig. 4). Similarly low values are apparent in the melting enthalpy (about 5 cal g^{-1}) and the temperature of transition III after extensive nuclease digestion, thus leading to solutions containing large fractions of mononucleosomes (Fig. 3) and dinucleosomes.

If the thermograms at low ionic strength are compared with those at physiological ionic strength, it is reassuring to note that for either chromatin preparation there is a similar enhancement, up to the value of native nuclei, of the melting enthalpy of transition II at 345 K, previously shown to be due to chromosomal proteins [16,21,22,35].

On further digestion with micrococcal nuclease (Fig. 7) at the same

TABLE 2

G0 chromatin melting enthalpies (cal g^{-1}) and transition temperatures (K) before and after shearing, at low and high ionic strength. Chromatin was prepared by the "nuclease" method or by the "cold water" method; for details see materials and methods

Method	Transition		
	II	III	IV
"Cold water", low ionic strength, gently suspended	0.53 ± 0.07 (340–350)	10.81 ± 4.8 (352–363)	6.29 ± 1.5 (364–375)
"Cold water", high ionic strength, gently suspended	4.76 ± 2.7 (340–351)	10.68 ± 4.1 (355–370)	10.06 ± 2.0 (371–384)
"Cold water", low ionic strength, vigorously suspended	0.13 ± 0.08 (340–350)	10.5 ± 3.4 (352–363)	–
"Cold water", high ionic strength, vigorously suspended	4.6 ± 2.5 (340–351)	9.0 ± 3.7 (352–370)	6.1 ± 2.7 (371–384)
"Cold water", low ionic strength, sheared	0.2 (340–350)	4.1 (352–368)	–
"Cold water", high ionic strength, sheared	1.0 (340–351)	4.0 (352–368)	–
"Nuclease", low ionic strength	0.05 ± 0.03 (340–350)	4.85 ± 1.8 (351–362)	–
"Nuclease", high ionic strength	4.1 (340–351)	9.2 (355–370)	–

concentration and time, chromatin prepared with the "cold water" method gives exactly the same pattern of bands as does a gel chromatin in intact nuclei, while chromatin prepared by the nuclease method lacks the DNA bands with higher molecular weight. Sheared chromatin displays only a broad smear of small DNA fragments, 1,000 base-pairs long or less, as previously shown [14].

However the systematic loss of the fourth transition at 375 K is not merely the effect of changes in DNA length per se (with the DNA high-order structure being the same), but it appears to be due to a loss of native higher order structure probably related to the loss of topological constraints caused by the reduction in DNA length. Indeed at a similar physiological ionic strength, an exact similarity is apparent in melting temperature and melting enthalpy (in cal g^{-1}) for free DNA fragments of quite different lengths,

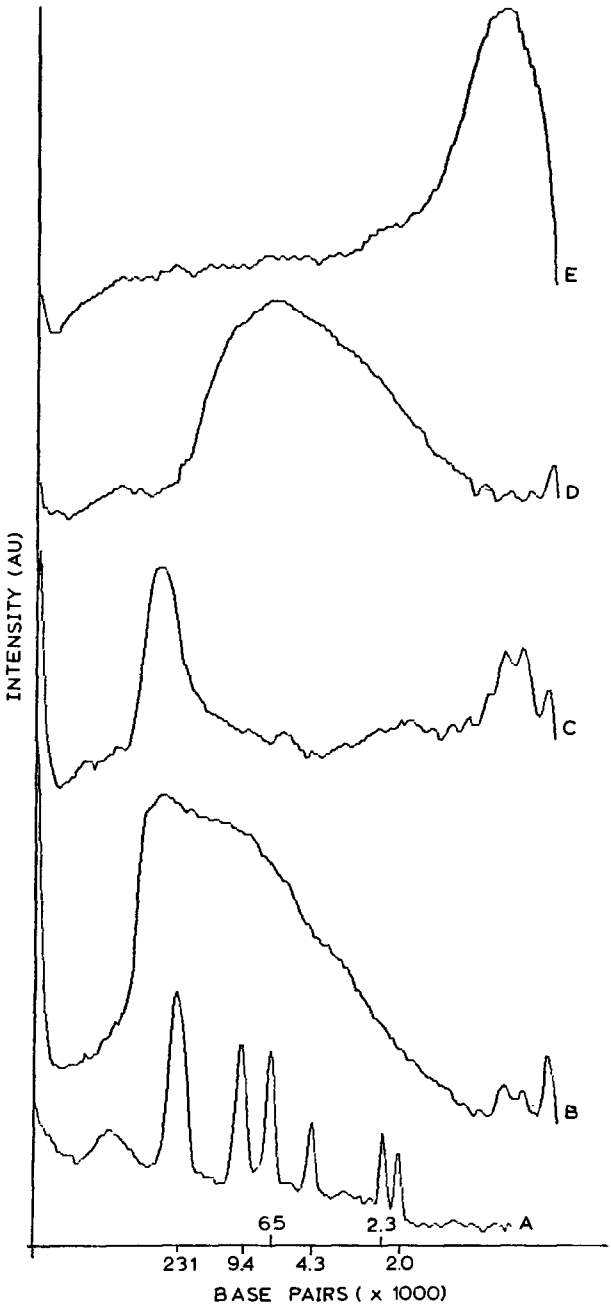

Fig. 7. As Fig. 3, but following digestion with 10 U ml^{-1} of micrococcal nuclease for 30 s of native nuclei (B), "nuclease" chromatin (D), "cold water" chromatin either unsheared (C) or sheared (E) and λ-DNA markers (A).

ranging from 30 kbp to 210 bp (Fig. 8, A and B). Even for a large DNA length, such as the one present in native nuclei, transition IV is consistently absent.

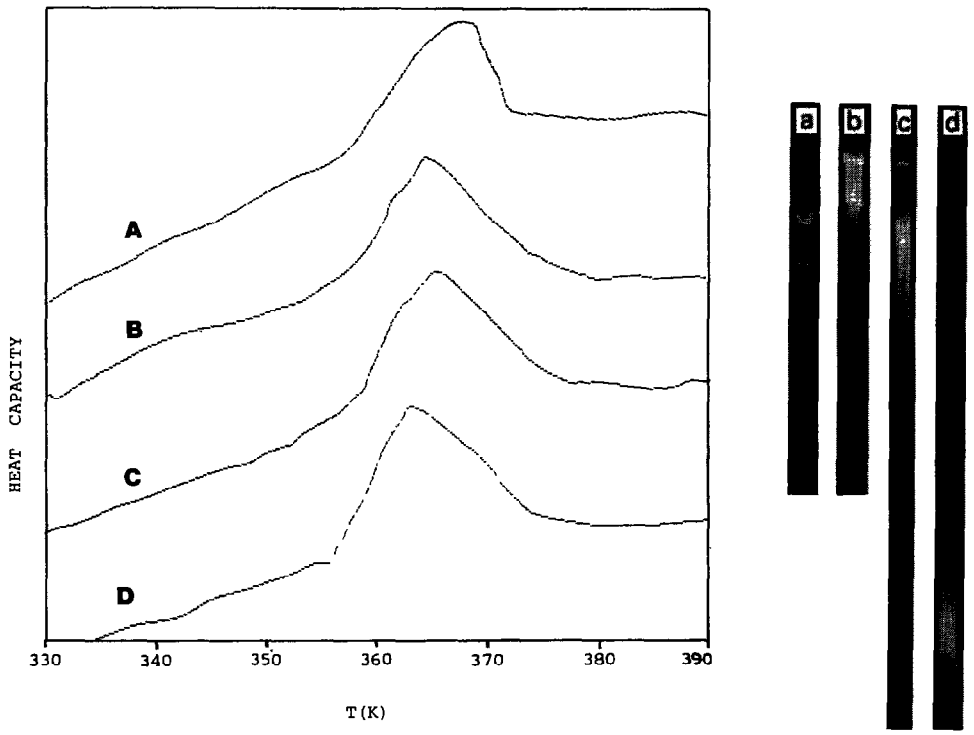

Fig. 8. Heat capacity profiles of free rat liver DNA intact (A), calf thymus sonicated (B), and intact (C), at 150 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris–HCl pH 8. The heat capacity profile of calf thymus DNA at 150 mM NaCl, 10 mM Tris–HCl pH 8 without $MgCl_2$ is shown for comparison (D). The corresponding gel electrophoretic profiles of the same DNA are shown: λ-DNA markers (a), rat liver DNA intact (b), calf thymus DNA intact (c), and sonicated (d).

This conclusion is supported, furthermore, by the fact that the dramatic changes that take place during cell cycle progression (Fig. 9) in the heat capacity profiles of the same unsheared chromatin prepared by the "cold water" method are not at all related to changes in the corresponding DNA length, because it remains constant (Fig. 10).

Following partial hepatectomy, chromatin from rat hepatocytes has been isolated and thermally characterized at 0, 18 and 24 h, corresponding to G0 phase, late G1–early S phase and G2–M phase respectively. High resolution autoradiography following ^3H thymidine pulse labelling shows that the percentage of cells at different stages of the cell cycle are respectively 100% in G0 at 0 h, 50% in G0 and 50% in late G1–S phase at 18 h, and 50% in G0 and 50% in late S–G2–M phase at 24 h [30]. The thermal profile at 18 h, compared with the one prior to hepatectomy (Fig. 9 and Table 3), shows at low ionic strength for truely native isolated chromatin an enhancement of the peak at 360 K at the expense of the peak at 372 K. Subsequently at 24 h, the thermogram shows a further redistribution between the transitions at

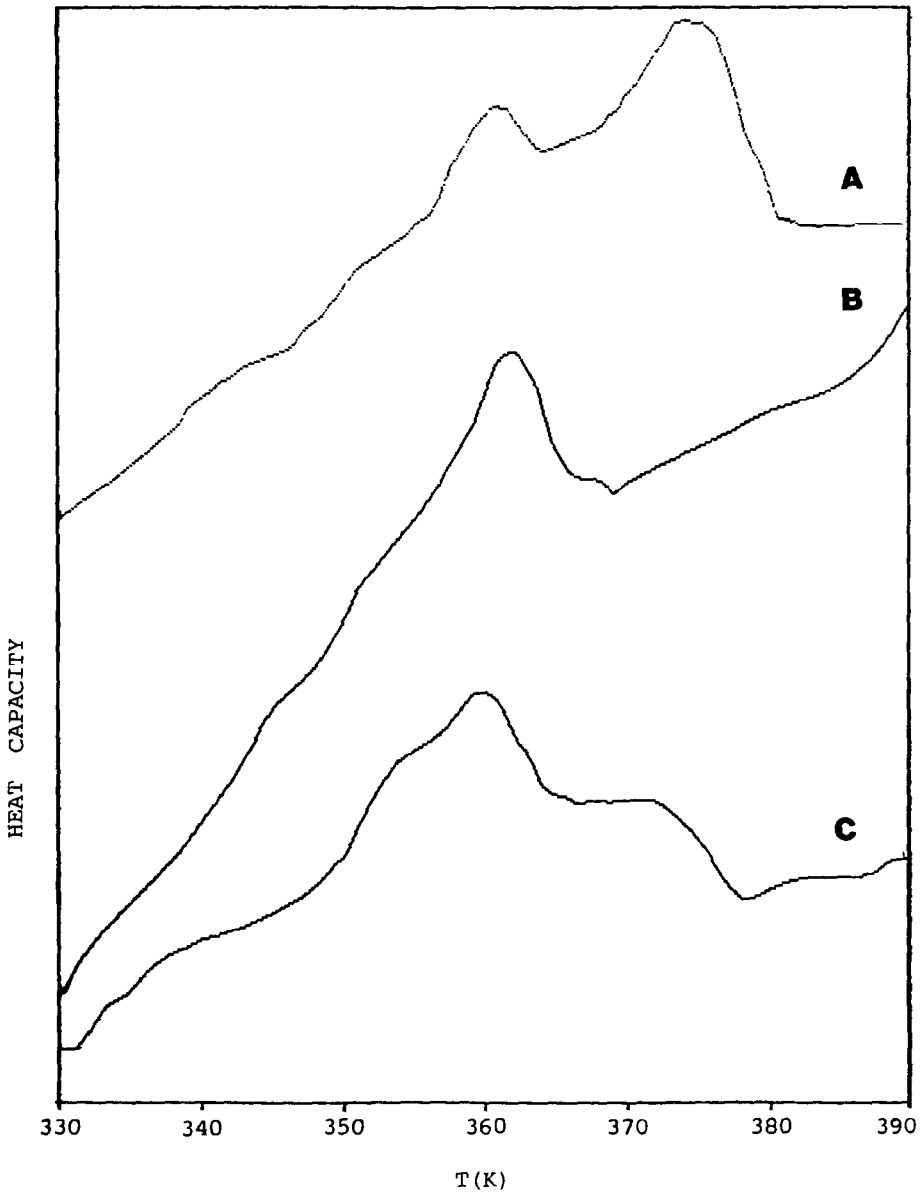

Fig. 9. Effect of cell proliferation on unsheared chromatin prepared by the "cold water" method: heat capacity (cal g^{-1}) versus temperature (K) of G0 (A), late G1–S (B) and G2–M (C) chromatin at low ionic strength.

360 and at 372 K, accompanied by an enhancement of the peak at 345 K which is parallelled by a sizeable increase in protein concentration (Fig. 9).

Cell-cycle-related alterations are absent in the heat capacity profiles of C0 and G1–S chromatin prepared by the nuclease method, which appear indistinguishable in the 352–380 K range (not shown).

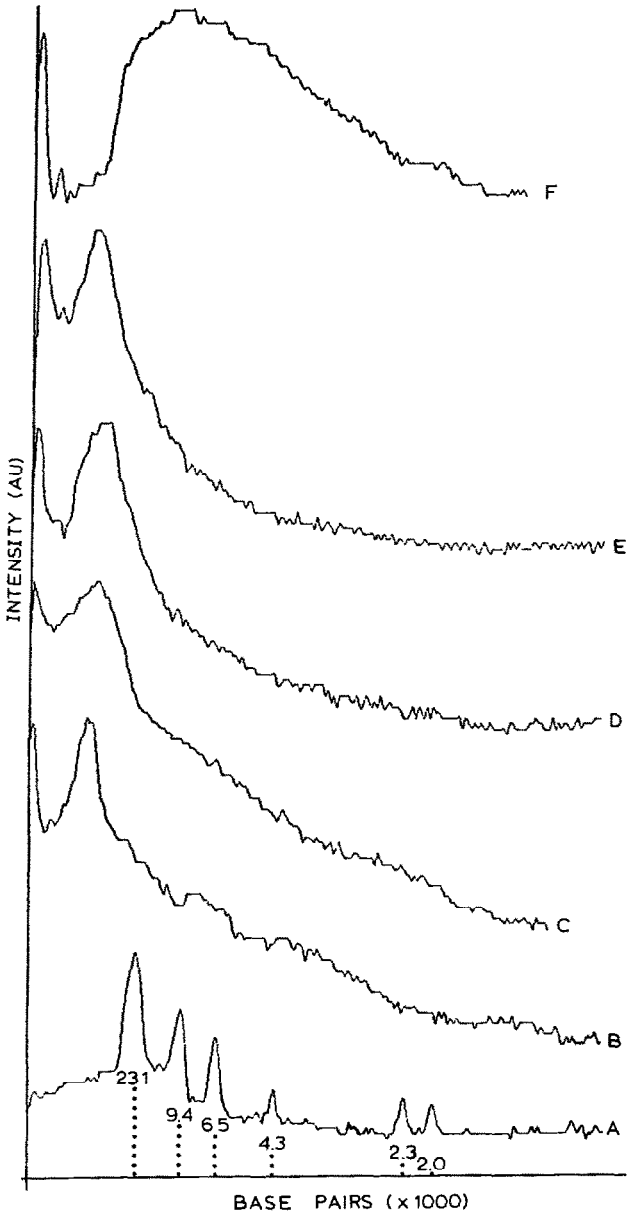

Fig. 10. As Fig. 3, but for λ-DNA markers (A), G0 native liver nuclei (B), "cold water" chromatin from G0 (C), G1–S (D) and G2–M (E) phase hepatocytes. The DNA size distribution of nuclei digested with 500 U ml^{-1} of DNA-ase D for 7 min is also given (F).

DISCUSSION

Despite the large variability (up to 20% on average) due to the sample preparations and the inherent limitation of calorimetric evaluations of a rather low heat capacity signal, our data on nuclei, chromatin and DNA

TABLE 3

Melting enthalpies (cal g^{-1}) and temperatures for the transitions (K) present in G0 at 10 mM Tris-HCl pH 8 for chromatin isolated by the "cold water" method from rat liver cells at 0, 18 and 24 h following partial hepatectomy

Time	Transition		
	II	III	IV
0 h	0.53 ± 0.07	10.81 ± 4.8	6.29 ± 1.5
	(336–349)	(350–363)	(364–375)
18 h	3.9 ± 0.023	8.2 ± 0.57	1.0 ± 0.01
	(336–349)	(350–367)	(368–380)
24 h	5.7 ± 0.11	12.7 ± 0.12	3.4 ± 0.03
	(336–349)	(350–367)	(368–380)

appear compatible with previous [16,21,22] assignments for the (at least) four major thermodynamic transitions, namely: quinternary chromatin–DNA structure, for transition I at 328 K; nuclear and chromosomal proteins, for transition II at 345 K; tertiary and secondary chromatin–DNA structure, for transition III at 360–365 K (depending on ionic strength); and quaternary chromatin–DNA structure, for transition IV at 372–375 K (depending on ionic strength).

The differences in the heat capacity profiles between nuclei mildly digested by DNA-ase and those mildly digested by micrococcal nuclease are likely to be related to the different action of the two enzymes with DNA-ase giving a higher proportion of single-strand breaks [31].

Based on the above, it should be noted that the damage to the native chromatin structure originates not only from shearing as previously shown [14,15], but also from the limited nuclease digestion itself, which causes the loss of topological constraints present in the native nuclei. These are, however, preserved in the corresponding unsheared chromatin prepared by the cold water method.

From a thermodynamic standpoint (melting enthalpy and temperature of transition III, and absence of transition IV), the nature of the fibre appears completely different in native chromatin, either in situ or when isolated by the "cold water" procedure, and in chromatin prepared by the nuclease method. Parallel measurements of circular intensity differential scattering [11] confirm the above conclusion suggesting that only "cold water" chromatin has a higher order structure at low ionic strength (due to the preservation of native possibly topological constraints) and that the conformational changes induced by increasing the ionic strength differ completely in the two chromatin preparations, with only the latter yielding the in situ structural properties.

Our findings could explain earlier electron micrography of chromatin in vivo [5,17] and are not at odds with earlier X-ray diffraction studies [7,8] of

living cells and isolated nuclei showing several major low-angle reflections, characteristic of a highly conserved chromatin packing within the fibres. The later studies lacked a rigorous quantitative comparison with dilute solution of chromatin prepared by limited digestion. Furthermore, controversy still surrounds the numerous X-ray studies of isolated chromatin.

The folding-back or twisting around each other of native filaments, which is seen in some form in metaphase chromosomes [11], has recently also been suggested for the relatively long 300 Å filament [3] and could well be a natural property of chromatin in vivo, allowable solely by topological constraints that are lost with limited nuclease digestion. These topological constraints could cause a higher degree of DNA supercoil, recently shown to be associated with a higher transition enthalpy [34].

Further evidence of the truely native nature of chromatin prepared by the "cold water" method is provided by the exact similarity between this chromatin preparation and native nuclei [22] in terms of their heat capacity profiles at various time intervals during synchronized cell cycle progression. This is per se a significant contribution to our understanding of native chromatin structure and its role in the control of DNA replication, gene expression and mitotic condensation [32]. Neutron and low-angle X-ray scattering techniques [33] have confirmed earlier observations obtained from high resolution image analysis [10], differential light scattering [12] and viscoelastometry [9] according to which chromatin inside the nucleus of G0 cells displays a higher order packing of the fibres which is absent in nuclei with higher gene activity [34] such as G1 or S phase cells. Our data are in perfect agreement with this, suggesting that initiation of DNA synthesis is associated with a significant increase in the fraction of genome undergoing transition from a quaternary structure to a tertiary nucleofilament-like structure. Further progression into G2–M phase causes an opposite structural transition towards the highly packed metaphase chromosomes (Fig. 9). The differences in thermal profile for a constant average size of the chromatin–DNA fibres prepared by the "cold water" method rule out any trivial effect of DNA length per se on the thermograms, as shown also by Fig. 8. It is reassuring that cell-cycle-related differences are undetectable on the heat capacity profiles of chromatin prepared by limited nuclease digestion (not shown), in compatibility with its apparent artefact nature.

In conclusion, most functional and structural studies have so far been conducted using chromatin prepared by the nuclease method and it seems unavoidable that many of these studies will need to be repeated, as they have no direct bearing on the structure and function of truely native chromatin–DNA in situ.

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