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# Organization of Transcribed Regions of Chromatin

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# Organization of transcribed regions of chromatin

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The endonuclease DNase II preferentially attacks a limited and tissue-specific portion of chromosomal DNA. This material may be separated from the bulk of chromatin DNA by virtue of its solubility in 2 mM $MgCl_2$. The $Mg^{2+}$ soluble fraction forms a specific subset of DNA sequences and is enriched four to sevenfold in sequences coding for cytoplasmic poly(A)-containing RNA and globin messenger RNA (in globin-producing cells). The bulk (70–90 %) of rapidly labelled RNA is found associated with the $Mg^{2+}$-soluble fraction.

Transcriptionally active, $Mg^{2+}$-soluble chromatin is organized into repeating subunits of DNA (200 ± 5 base pairs) and histone. $Mg^{2+}$-soluble active subunits differ from the subunits or nucleosomes of non-transcribed regions in many respects: namely, chemical composition (non-histone protein and RNA), sedimentation properties, differential sensitivity to DNase I and the single-strand-specific nuclease S1, and optical melting behaviour. These results suggest that chromatin subunits adopt a new configuration during the process of transcription.

## Introduction

The genetic material of eukaryotes is organized into regular repeating nucleoprotein subunits which contain about 200 base pairs of DNA in association with an octomer of histone (Kornberg 1974; for review see Elgin & Weintraub 1976). Evidence for the subunit structure of chromatin comes from nuclease digestion studies (Hewish & Burgoyne 1973; Noll 1974*a*; Sahasrabuddhe & Van Holde 1974; Sollner-Webb & Felsenfeld 1975; Axel 1975), from chemical cross-linking experiments (Kornberg & Thomas 1974), and from direct observation of nucleoprotein particles in the electron microscope (Olins & Olins 1974; Oudet, Gross-Bellard & Chambon 1975; Finch, Noll & Kornberg 1975). Chromatin subunits have been termed ν-bodies (Olins & Olins 1974) or nucleosomes (Oudet *et al.* 1975). Certain questions may now be asked about the organization of DNA in nucleosomes: just how much nuclear DNA is contained in nucleosomes and what types of sequences are present? First, over 85 % of chromatin DNA can be recognized in the nucleosome repeat pattern after a brief micrococcal nuclease digest (Noll 1974*a*). Secondly, no major kinetic class of DNA sequences is absent from monomeric nucleosomes; the reassociation curves of nucleosomal DNA and total DNA are identical (Lacy & Axel 1975). Furthermore, both transcribed and non-transcribed DNA sequences are found in nuclease-resistant structures. Non-transcribed satellite DNA sequences (Lipchitz & Axel 1976) as well as messenger RNA-coding sequences (Lacy & Axel 1975; Kuo, Sahasrabuddhe & Saunders 1976) are found in nucleosomal DNA. Thus the mere association of histone with DNA is not sufficient to restrict transcription.

Cytological data, however, suggest that chromosomal DNA engaged in transcription is structurally different from non-transcribed DNA. Transcription takes place in the extended segments of chromatin: the puffs of insect polytene chromosomes (Barendes 1973), the lateral loops of amphibian lampbrush chromosomes (Callan & Lloyd 1960) and the diffuse euchromatic regions of interphase nuclei (Littau, Allfrey, Frenster & Mirsky 1964). Chromatin



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condensation, on the other hand, is associated with a loss of transcriptional activity. Isolated chromatin retains many structural differences between active and inactive regions. First, prokaryotic RNA polymerase recognizes a restricted number of initiation sites on chromatin as compared with deproteinized DNA (Cedar & Felsenfeld 1973), and transcription *in vitro* is to some degree tissue-specific (Axel, Cedar & Felsenfeld 1973; Gilmour & Paul 1973). Secondly, active genes are highly sensitive to attack by two nucleases, pancreatic DNase I (Weintraub & Groudine 1976; Garel & Axel 1976; Berkowitz & Doty 1975) and spleen DNase II (Gottesfeld *et al.* 1974, 1976). These data suggest that the DNA of active chromatin is in a more open or accessible conformation than the DNA of transcriptionally inactive regions.

Since structural differences between active and inactive regions of chromatin must have a biochemical basis, it should be possible to purify active segments of chromatin. Over the past decade many authors have described fractionation schemes and presented evidence to show that the isolated fractions of chromatin correspond to active regions *in vivo*. In order to separate active from inactive segments, chromatin DNA must be reduced to lengths shorter than the average unit of transcription. Most reported procedures start with sonicated or pressure-sheared chromatin, and fractionation is achieved by either differential centrifugation (Frenster, Allfrey & Mirsky 1963; Murphy, Hall, Shepherd & Weiser 1973; Berkowitz & Doty 1975), or by chromatography (McConaughy & McCarthy 1972; McCarthy *et al.* 1973; Simpson & Reeck 1973), or by buoyant density centrifugation (Rickwood *et al.* 1974). These procedures produce fractions with properties expected for active chromatin; the active fractions contain nascent RNA chains and have high template activities *in vitro*. However, upon closer examination of the DNA of the active fractions produced by four methods (ECTHAM cellulose chromatography, glycerol gradent centrifugation, gel exclusion chromatography and buoyant density centrifugation) no enrichment for transcribed sequences has been observed (Howk *et al.* 1975; Krieg & Wells 1976; Rickwood *et al.* 1974).

These findings demonstrate that copurification of RNA and high template activity are insufficient criteria to assess the validity of a fractionation procedure. The following criteria must be satisfied before any fractionation is to be judged successful: first, since the portion of the genome which is actually expressed *in vivo* is limited, the amount of DNA recovered in the active fraction should be small and should correspond to the fraction of DNA which is transcribed in the cell type under study. Secondly, the DNA of the active fraction should be a specific subset of the genome rather than a random population of sequences. Ideally, the sequence complexity of active fraction DNA should be equivalent to the mass fraction of the genome recovered. Thirdly, the active fraction must be enriched in transcribed sequences. DNA sequences complementary to specific messenger RNAs should be found in high concentration in the active fraction of transcribing cells but not in the active fraction of non-transcribing cells.

## A procedure for chromatin fractionation

Since the work of Noll, Thomas & Kornberg (1975), serious doubt must be cast on the results of fractionation procedures with sheared chromatin. Noll *et al.* (1975) report that native nucleosome structures are damaged by mechanical shear, and hence procedures such as homogenization, sonication or pressure shearing must be avoided. This work and previous studies (Marushige & Bonner 1971; Billing & Bonner 1972; Bonner *et al.* 1973; Gottesfeld

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*et al.* 1974, 1976) have used the endonuclease DNase II to cleave chromatin DNA. Fractionation is achieved in two ways: first, DNase II appears to attack transcribed regions of chromatin more rapidly than inactive regions (Gottesfeld *et al.* 1976) and, secondly, inactive chromatin may be removed from solution by precipitation with divalent cations. The basis for the solubility properties of active chromatin will be discussed later.

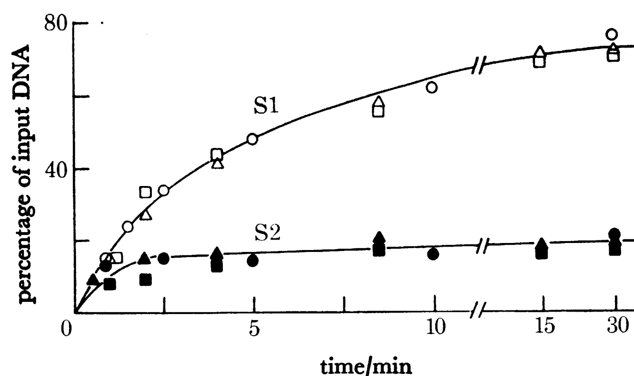


FIGURE 1. Time course of chromatin fractionation. Rat liver nuclei were lysed by suspension in 0.2 mM EDTA, pH 7, and sodium acetate was added to a final concentration of 25 mM (pH 6.6). After various times of incubation with DNase II (at 4 units/$A_{260}$ unit of DNA), samples were withdrawn and added to an appropriate volume of 0.1 M Tris–Cl (pH 8) to give a final pH of 7.5. Separation of chromatin into the first supernatant fraction (S1) and subsequent fractionation into $Mg^{2+}$-soluble (S2) and insoluble material was achieved as described in the text. Aliquots of each fraction were taken for estimation of DNA content by absorption at 260 nm in 0.1 M NaOH. Open symbols, first supernatant (S1); filled symbols, $Mg^{2+}$-soluble fraction (S2). Circles, squares and triangles represent independent experiments.

We start the fractionation procedure with either chromatin purified by sucrose gradient centrifugation (Marushige & Bonner 1966) or with chromatin in the form of gently lysed nuclei (Noll *et al.* 1975). Chromatin is treated with DNase II (at 2–10 enzyme units per $A_{260}$ unit) for various lengths of time at pH 6.6. The reaction is stopped by raising the pH to 7.5 with 0.1 M Tris–Cl, pH 8, and the sample is centrifuged (10000 *g* for 10 min). Centrifugation yields a supernatant of solubilized chromatin (termed S1) and a pellet (P1). The supernatant is further fractionated by the addition of $MgCl_2$ to 2 mM. The precipitate that forms is removed from solution by centrifugation (10000 *g* for 10 min). The second pellet fraction is termed P2 and the final supernatant of $Mg^{2+}$-soluble chromatin is termed S2.

Figure 1 shows a time course of digestion of chromosomal DNA in gently lysed rat liver nuclei. After an initial rapid rise in the appearance of soluble material (S1), the rate of appearance falls progressively. On long times of digestion, 70–80 % of chromatin DNA is rendered soluble. These data suggest the existence of several classes of chromatin regions which differ in degree of susceptibility to DNase II. When the first supernatant is fractionated into $Mg^{2+}$-soluble (S2) and $Mg^{2+}$-insoluble material (P2), we see that $Mg^{2+}$-soluble material forms part of the most nuclease-sensitive fraction of chromatin DNA (figure 1). It has been noted previously (Billing & Bonner 1972) that the amount of chromatin DNA recovered in the $Mg^{2+}$-soluble fraction is variable, depending upon the transcriptional activity of the cell type under investigation. Active tissues, such as liver and brain, and Friend erythroleukaemia cells, yield 17–25 % of their DNA in the $Mg^{2+}$-soluble chromatin fraction. Inactive tissues, such as chicken erythrocytes, yield only 2–5 % of their DNA in this fraction (Woodcock, Sweetman &



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Frado 1976; R. Axel and G. Felsenfeld, personal communication). Thus the amount of DNA recovered in the $Mg^{2+}$-soluble fraction appears to correlate with the extent of transcription in the cell type under study.

## Nucleic acid sequence composition of active chromatin

The reassociation kinetics of DNA from unfractionated chromatin and $Mg^{2+}$-soluble (S2) chromatin have been determined (figure 2). Both DNAs exhibit fast, intermediate and slow renaturing components; for both total and $Mg^{2+}$-soluble chromatin these components represent 10%, 25% and 65% of the input DNA, respectively. We do not know whether the fast-reassociating component of S2 DNA is composed of fold-back sequences or highly repetitive

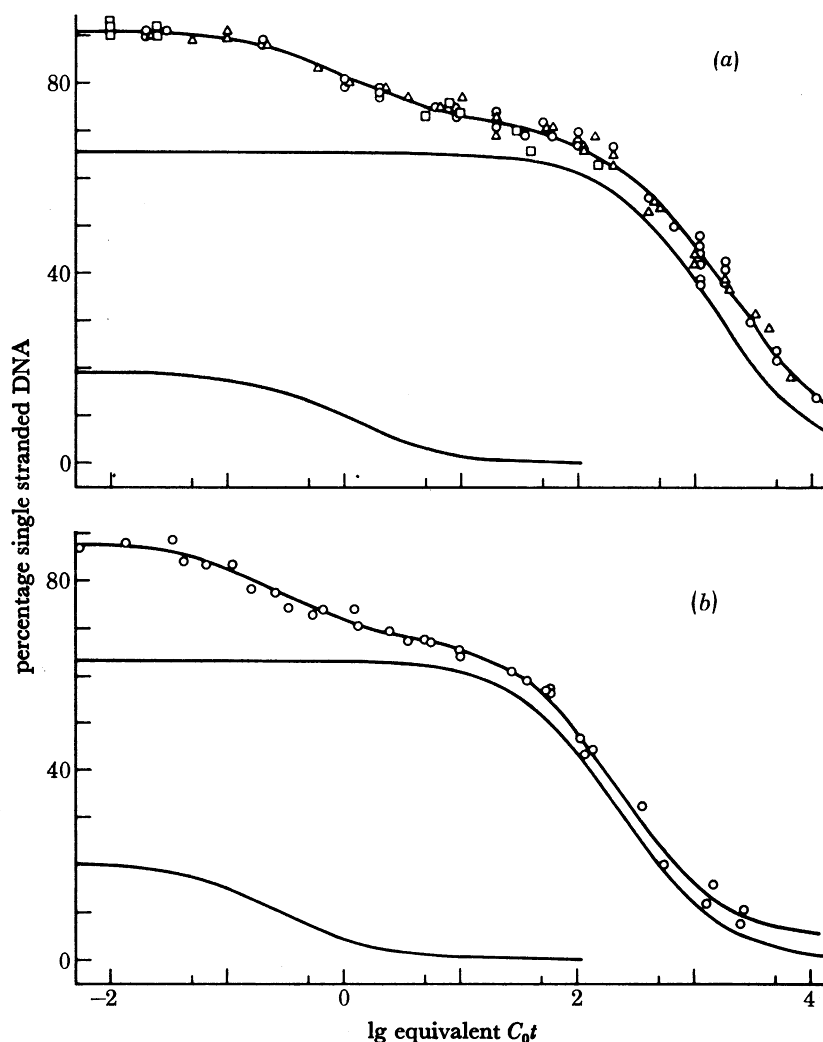


Figure 2. Reassociation profiles of (*a*) unfractionated chromatin DNA and (*b*) DNA isolated from fraction S2 of rat liver. Renaturation was assayed by hydroxyapatite chromatography (Britten, Graham & Neufeld 1974). DNA from unfractionated chromatin was sheared to 350–400 base pairs by two passes through a Ribi-Sorval pressure cell at *ca.* 340 MPa (50000 lbf/in²). Fraction S2 was isolated as described (Gottesfeld *et al.* 1974). The data have been normalized to $C_0t$ values expected for 0.18 M $Na^+$ (0.12 M phosphate buffer) and the lines through the data were obtained by a two-component least-squares computer analysis (Britten *et al.* 1974). The solid lines represent the component curves.

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sequences. The intermediate and slow components of S2 DNA renature at rates seven to ten times faster than their respective counterparts in unfractionated chromatin DNA. The $C_0t_{\frac{1}{2}}$ values for the slow components of total DNA and S2 DNA are 2000 and 225, respectively. This difference in renaturation rate is due neither to different DNA fragment lengths nor to reaction conditions. Previous studies have shown that the slow component of S2 DNA is complementary to non-repetitive sequences in total rat DNA (Gottesfeld *et al.* 1974). Thus S2 DNA is composed of a limited fraction (about 10%) of the complexity of total rat DNA; that is, S2 DNA is a specific subset of the genome rather than a random sample of DNA sequences. This holds true for both repeated and single-copy sequences. The intermediate component of S2 DNA renaturation is composed of a subset of families of moderately repetitive sequences (Gottesfeld *et al.* 1976). This latter finding demonstrates the non-random distribution distribution of repetitive and unique sequences in the eukaryotic genome.

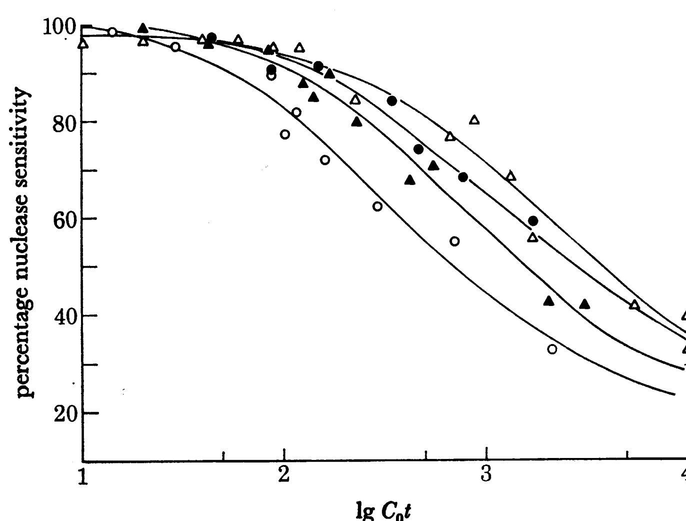


Figure 3. Annealing kinetics of radioactive globin cDNA in the presence of an excess of unlabelled DNA from chromatin fractions. Chromatin from either dimethylsulphoxide-induced or non-induced Friend cells (clone M2), or from adult mouse liver nuclei, was fractionated as described in the text, and DNA was prepared from the chromatin fractions. P1 DNA was sheared to a mass-average length of 300–400 nucleotides by sonication (six bursts of 15 s each). Globin cDNA (8000–20000 count/min) was mixed with the DNA preparations and the samples were precipitated with ethanol. Precipitates were collected by centrifugation, dried under vacuum and dissolved in 200 μl of 0.6 M NaCl, 1 mM Tris–Cl, 0.25 mM EDTA, pH 8. Annealing reactions were carried out at 66 °C in sealed siliconized capillary tubes. $C_0t$ values have been normalized to 0.18 M $Na^+$. The extent of annealing was estimated by the micrococcal nuclease assay (Kacian & Spigelman 1974). The solid lines represent the best least-squares computer analyses of the data. Globin cDNA annealing in the presence of: ○, S2 DNA from dimethylsulphoxide-induced Friend cells at 1.1 mg DNA/ml, $C_0t_{\frac{1}{2}}$ = 370; ▲, S2 DNA from non-induced Friend cells at 1.6 mg/ml, $C_0t_{\frac{1}{2}}$ = 880; ●, S2 DNA from mouse liver at 0.6 mg/ml, $C_0t_{\frac{1}{2}}$ = 1300; and △, P1 DNA from induced Friend cells at 1.5 mg/ml, $C_0t_{\frac{1}{2}}$ = 2100.

## Transcribed sequences in $Mg^{2+}$-soluble chromatin DNA

From DNA–RNA hybridization experiments it was shown that S2 DNA is enriched four- to sevenfold in non-repetitive sequences complementary to total cellular RNA from rat liver (Gottesfeld *et al.* 1974). Since the major fraction of the complexity of cellular RNA is due to sequences confined to the nucleus (Davidson & Britton 1973), these data do not indicate whether messenger RNA-coding sequences are found in S2 DNA. To investigate this matter

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we have used Friend erythroleukaemia cells (Gottesfeld & Partington 1977). Friend cells grown under standard conditions of cell culture synthesize no detectable amounts of haemoglobin protein (Ross *et al.* 1974) and only low levels of globin messenger RNA (about 100 copies per cell cytoplasm). Upon treatment with dimethylsulphoxide (1.5 % for 3 days in culture) Friend cells are induced to synthesize large quantities of globin mRNA (1500–5000 copies per cell) and globin protein. The relative concentrations of DNA sequences complementary to globin cDNA have been determined for the chromatin fractions produced by the DNase II/$Mg^{2+}$-solubility procedure (figure 3). The rate of globin cDNA annealing to either S2 DNA from non-stimulated Friend cells or to S2 DNA from mouse liver is the same as the annealing rate to total DNA, indicating no enrichment for globin sequences in these S2 fractions. S2 DNA from dimethylsulphoxide-stimulated cells, however, is enriched in globin nucleotide sequences. Globin sequences are six times more abundant in S2 DNA than in P1 DNA from stimulated cells.

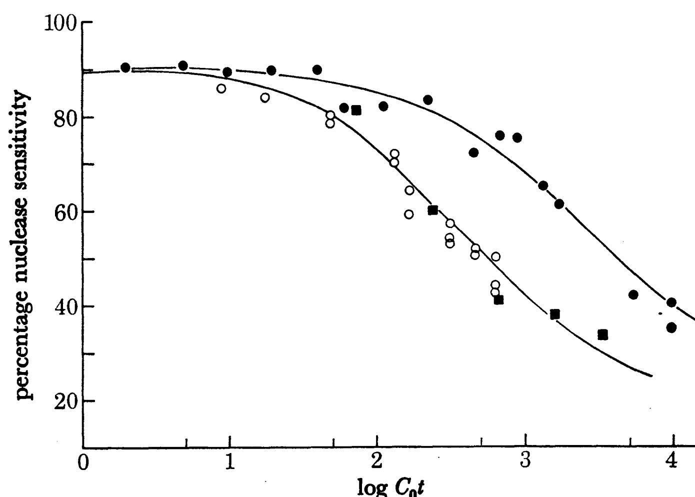


FIGURE 4. Annealing of Friend cell (PolyA+) cDNA to DNA from chromatin fractions. Friend cells were grown for 3 days in the presence of 1.5 % (by volume) dimethylsulphoxide and chromatin was fractionated as described in the text. DNA was prepared from fractions S2 and P1 by standard techniques and P1 DNA was sheared to 300–400 nucleotides by sonication. A count of 50000/min (*ca.* $1 \times 10^{-2}$ μg) of a cDNA to Friend cell poly(A)-containing RNA were mixed with each of these DNA samples (1.6 mg of P1 DNA and 0.64 mg of S2 DNA). The samples were precipitated with ethanol and the precipitates were collected by centrifugation. After drying, the pellets were dissolved in 0.48 M PB. An aliquot of each sample was diluted with distilled water to give 0.24 M PB for low $C_0t$ points. Annealing reactions were carried out at 66 °C (0.48 M PB) and at 62 °C (0.24 M PB). $C_0t$ values were normalized to standard conditions (0.18 M $Na^+$). The solid lines describe the best least-squares fit of the experimental data: ○, ■, S2 DNA (two experiments), $C_0t_{\frac{1}{2}} = 310$; ●, P1 DNA, $C_0t_{\frac{1}{2}} = 1900$.

The cytoplasmic poly(A)-containing RNA of Friend cells comprises a diverse range of nucleotide sequences: there are a few sequences present many thousand times per cell as well as several thousand sequences present only in limited numbers of copies. The total complexity of Friend cell poly(A)-containing cytoplasmic RNA is equivalent to about 14000 different sequences of average length (Gottesfeld & Partington 1977). DNA sequences coding for these poly(A)-containing RNAs are more abundant in the $Mg^{2+}$-soluble fraction of Friend cell chromatin than in the pellet fractions (figure 4). From cDNA annealing experiments, there is a sixfold enrichment in DNA sequences complementary to poly(A)-containing RNA in S2 DNA.

Thus the active fraction isolated by the DNase II/$Mg^{2+}$-solubility procedure satisfies the

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criteria listed above. The amount of DNA recovered in the $Mg^{2+}$-soluble fraction correlates well with the transcriptional activity of the cell type under study. The DNA sequences of the active fraction form a specific subset of the genome rather than a random sample of sequences, and this fraction is enriched in transcribed sequences. Furthermore, a specific mRNA sequence (globin) is found in high abundance in the $Mg^{2+}$-soluble active fraction of transcribing cells, but not in high abundance in the active fraction of non-transcribing cells.

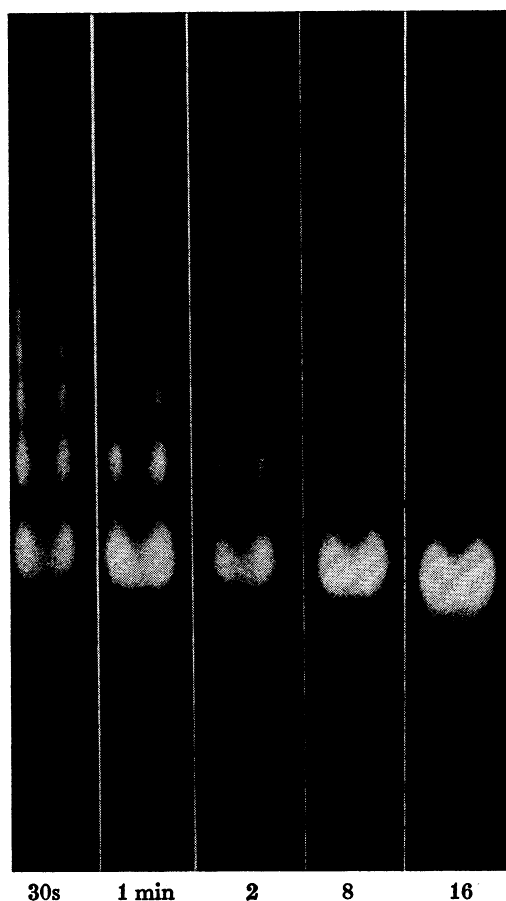


Figure 5. Agarose gel electrophoresis of $Mg^{2+}$-soluble chromatin DNA. Rat liver nuclei were lysed and treated with DNase II (4 units/$A_{260}$ unit) as described in the text. At each of the times indicated an aliquot of the chromatin suspension was withdrawn and fractionated. Purified DNA from fraction S2 (approximately 5–10 μg per sample) was electrophoresed for 14 h at 24 mA in a 2% agarose gel. After electrophoresis the gel was stained with ethidium bromide (0.5 μg/ml) and photographed.

## Repeating units in $Mg^{2+}$-soluble active chromatin

The size of the DNA in the $Mg^{2+}$-soluble fraction of rat liver chromatin has been investigated previously by electron microscopy (Gottesfeld *et al.* 1976). The longest DNA fragments observed were about 2000 base pairs in length. As digestion time was increased, the length of $Mg^{2+}$-soluble chromatin DNA decreased progressively. Figure 5 shows the results obtained when S2 DNA, isolated after various times of DNase II digestion, is electrophoresed in 2% agarose gels. At early times of digestion a typical band pattern is observed. A semi-logarithmic plot of distance of migration against lg (band number) reveals that the higher bands are integral

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multiples of a unit length. As digestion proceeds, the DNA passes from multimers into a monomer band. The size of the monomer band appears to decrease with increasing times of digestion, presumably due to shortening of the DNA from the ends by further nuclease digestion.

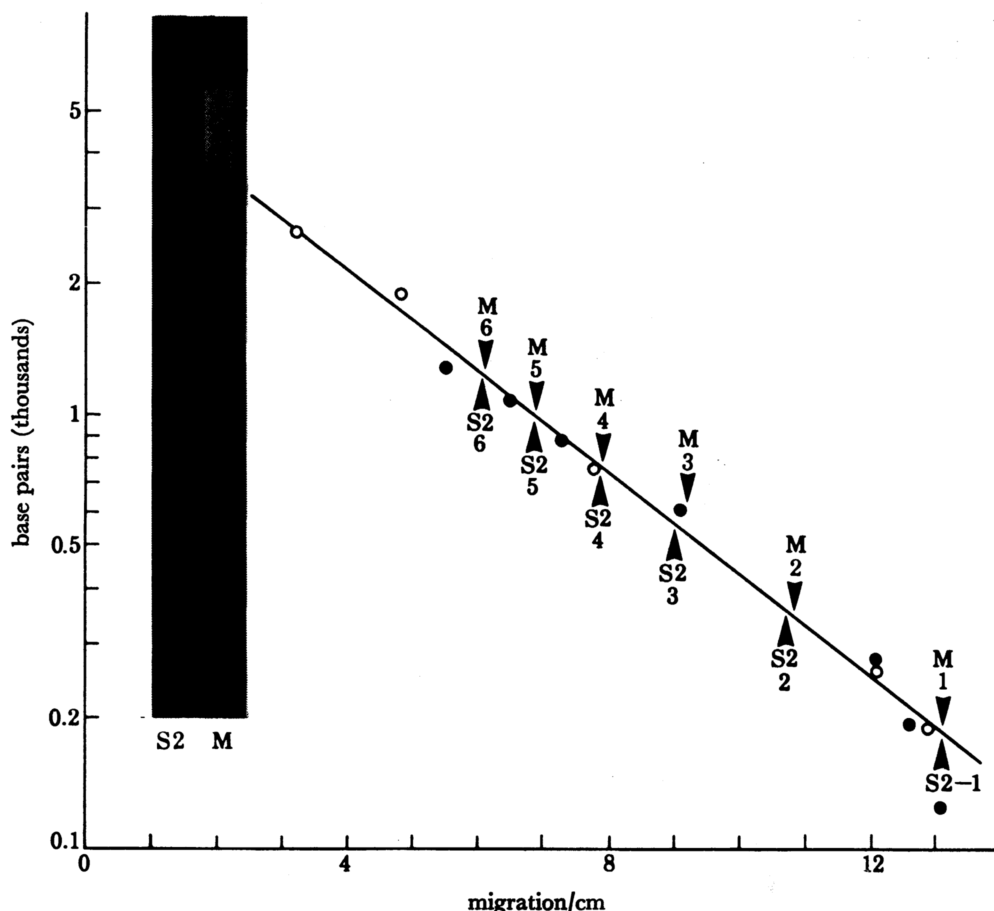


FIGURE 6. Determination of the size of DNA fragments from nuclease digestions of chromatin. Rat liver nuclei were treated with DNase II (4 units/$A_{260}$ unit for 1 min) and the chromatin was fractionated as described. Nuclei were also treated with micrococcal nuclease (100 units/ml for 3 min at 37 °C) with no subsequent fractionation. DNA was purified from the micrococcal nuclease treated nuclei and from the $Mg^{2+}$-soluble fraction (S2) of the DNase II treated chromatin. Aliquots (10 μg) of each DNA sample were electrophoresed for 18 h at 30 mA in a 2% agarose gel. Sequenced restriction fragments (Hae II and Hae III) of ϕX 174 RF DNA were used for size determination. ○, Hae II fragments; ●, Hae III fragments: M1–M6, position of micrococcal nuclease generated chromatin DNA fragments, monomer to hexamer; S21 to S26, position of DNase II generated fragments of $Mg^{2+}$-soluble chromatin DNA, monomer to hexamer. Gel: S2, $Mg^{2+}$-soluble chromatin DNA produced by DNase II digestion; M, micrococcal nuclease digest.

To obtain an accurate estimate of the DNA repeat length in S2 chromatin, DNA isolated after 1 min of DNase II digestion was coelectrophoresed with Hae II and Hae III restriction fragments of ϕX 174 RF DNA (figure 6). The lengths of the chromatin DNA fragments were determined from a least-squares regression analysis of the logarithm of restriction fragment length plotted against distance of migration. As the length of monomer DNA might be greatly affected by exonucleolytic attack, the repeat length was determined by taking the difference in length between successive multimer bands. From this analysis the DNA repeat is $199 \pm 6$ base pairs, a value identical to the repeat length for a micrococcal nuclease digest of unfraction-

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ated nuclear chromatin (198 ± 5 base pairs; figure 6). Thus it appears that the organization of $Mg^{2+}$-soluble, transcriptionally active chromatin DNA is similar to the organization of bulk transcriptionally dormant DNA.

Repeating subunits of nucleoprotein have been demonstrated by electron microscopy (Olins & Olins 1974; Woodcock 1973; Oudet *et al.* 1975) and by sedimentation of nuclease-treated chromatin in sucrose gradients (Noll 1974*a*; Lacy & Axel 1975). Noll (1974*a*) reports that monomer, dimer and trimer nucleosomes exhibit $S_{20,w}$ values of 11.2 ± 0.4, 15.9 ± 0.5 and 21.5, respectively. The nucleoprotein subunits of $Mg^{2+}$-soluble chromatin sediment somewhat faster than this (figure 7). From 28 determinations similar to that shown in figure 7, the monomer subunit of $Mg^{2+}$-soluble chromatin exhibits an $S_{20,w}$ value of 14.0 ± 0.8. The dimer

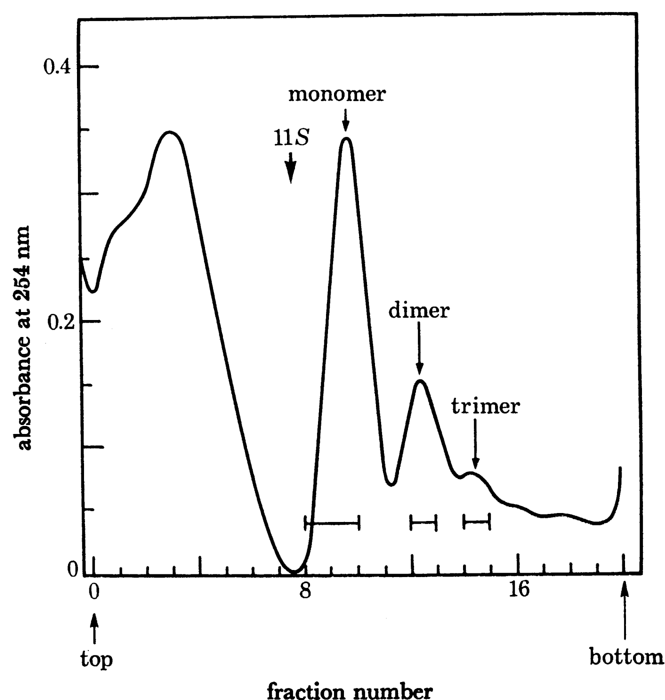


FIGURE 7. Sucrose gradient sedimentation of $Mg^{2+}$-soluble (S2) chromatin. Rat liver nuclei were prepared, treated with DNase II (10 units/$A_{260}$ unit for 1.5 min) and the chromatin was fractionated as described. The $Mg^{2+}$-soluble fraction (S2) was concentrated 3.3-fold with an Amicon Minicon device. 1.5 ml of this material were layered on a 5–24% (by mass) isokinetic sucrose gradient containing 10 mM Tris–Cl (pH 8). Centrifugation was at 35000 rev/min for 15 h in the SW40 rotor. The gradient was fractionated with an M.S.E. device equipped with an Isco u.v. absorbance monitor. Fractions were pooled as indicated.

TABLE 1. CHEMICAL COMPOSITION OF RAT LIVER CHROMATIN AND CHROMATIN SUBUNITS

| | composition relative to DNA (by mass) | | |
|---|---|---|---|
| | histone | non-histone | RNA |
| unfractionated chromatin | 1.06 | 0.65 | 0.05 |
| 11*S* subunits | 1.03 | 0.05 | < 0.05 |
| $Mg^{2+}$-soluble subunits | | | |
| 14*S* monomer | 0.97 | 1.35 | 0.3–0.4 |
| 18*S* dimer | 0.81 | 3.2 | 0.3–0.7 |

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sediments at $18.7 \pm 1.0S$. DNA isolated from the $14S$ peak is of monomer length; DNA from the $18S$ nucleoprotein peak is of dimer length, and so on (data not shown). The optical density observed at the top of the sucrose gradient depicted in figure 7 is due to light scattering by nuclear debris. Very little acid soluble material (less than 3–5 % of the total DNA) is produced during the brief nuclease digestions (1–2 min). The sedimentation values reported above were obtained with isokinetic sucrose gradients (Noll 1967) using $11S$ nucleosomes as a marker. If the $14S$ subunit of $Mg^{2+}$-soluble chromatin has a different density from that of the $11S$ nucleosome, these $S_{20,w}$ values could be in error. On sedimentation in the analytical ultracentrifuge, $14S$ subunits of $Mg^{2+}$-soluble chromatin exhibited an $S_{20,w}$ value of $15.4 \pm 0.3$.

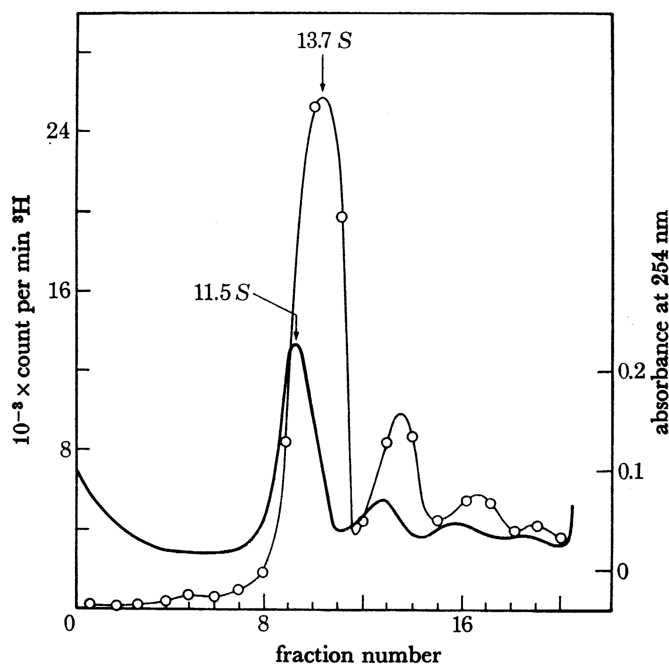


FIGURE 8. Sucrose gradient sedimentation of a micrococcal nuclease digest of [$^3$H]uridine labelled HeLa nuclei. HeLa cells were grown on the surface of a 20 ml culture flask for 2 days before the addition of [$^3$H]uridine (at a final concentration of 20 μCi/ml in 10 ml of medium). After 1 h of incubation at 37 °C the medium was discarded. 10 ml of balanced salts solution were added and the cells were dislodged from the walls of the flask with a rubber 'policeman'. The cells were centrifuged at 2500 rev/min for 5 min and the pellet was suspended in 2.5 ml of isotonic buffer containing 0.4 % NP-40. The cells were incubated at 0 °C for 10 min to ensure lysis. The sample was centrifuged and washed in an additional 1.25 ml of isotonic buffer. After centrifugation the nuclear pellet was suspended in 1 ml of 0.34 M sucrose-buffer A with 3 mM $CaCl_2$ (Noll *et al.* 1975). Micrococcal nuclease was added to 200 units/ml and incubation was at 37 °C for 4 min. The reaction was stopped by the addition of EDTA (pH 8) to 25 mM, and the nuclei were lysed as described. The nuclear lysate was centrifuged on a 5–24 % isokinetic sucrose gradient at 35000 rev/min for 14.75 h. Aliquots of each fraction were precipitated with 10 % trichloroacetic acid. Insoluble material was collected on filters and counted in Liquifluor. ——, Optical density profile; —o—, trichloroacetic acid-insoluble radioactivity.

The chemical compositions of rat liver chromatin and chromatin subunits are given in table 1. Both $11S$ nucleosomes and $14S$ subunits contain a full complement of histone protein; furthermore, no species of histone is absent from the subunits of $Mg^{2+}$-soluble chromatin when care is taken to prevent proteolysis (Gottesfeld, Murphy & Bonner 1975). The major difference between $Mg^{2+}$-soluble $14S$ subunits and $11S$ nucleosomes is the high concentration of non-histone protein and RNA in the $Mg^{2+}$-soluble subunits. Polyacrylamide gel electrophoresis

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reveals a complex pattern of proteins, suggesting that the subunits of $Mg^{2+}$-soluble chromatin must be heterogeneous in composition (Gottesfeld *et al.* 1975). Pederson & Bhorjee (1975) have suggested that many of the non-histone proteins of $Mg^{2+}$-soluble chromatin are RNA-binding proteins.

On occasion, sucrose gradient-purified 14*S* subunits exhibited $S_{20,w}$ values in the range 10.7–11.6*S* on sedimentation in the analytical ultracentrifuge. On one occasion, two sedimenting components (11*S* and 14–15*S*) were observed. If 14*S* subunits are treated with RNase (10 μg/ml for 10 min at 37 °C) and then resedimented in an isokinetic gradient, the RNase-treated material sediments at 11*S*. Control 14*S* material resediments as before, at 14*S*. Furthermore, the 11*S* material obtained from RNase treatment of 14*S* subunits is insoluble in 2 mM $MgCl_2$. The 11*S* material produced by RNase treatment has a molecular mass of 190000–209000 (as determined by low speed sedimentation equilibrium centrifugation), a value identical to the molecular mass of authentic 11*S* nucleosomes. Thus the basis for fractionation with $MgCl_2$ appears to be the association of an RNA–protein complex with the nucleosomal DNA of active chromatin.

## Purification of nascent RNA with 14*S* subunits

Bonner *et al.* (1973) and Kimmel, Sessions & McCleod (1976) have reported that the major portion of rapidly labelled RNA bound to chromatin is localized in the $Mg^{2+}$-soluble fraction. To investigate the possibility that the RNA found in the 14*S* subunits of $Mg^{2+}$-soluble chromatin might be newly transcribed RNA, the experiment of figure 8 was performed. HeLa cells were labelled *in vivo* with [$^3$H]uridine and nuclei were prepared. The nuclei were treated with micrococcal nuclease and the chromatin was analysed in an isokinetic sucrose gradient. While the monomer nucleosome peak sedimented at 11.5*S*, the first peak of radioactivity was at 13.7*S*. Similarly, the 'multimers' of radioactivity sedimented faster than the multimers of optical density. Micrococcal nuclease was used for this experiment as the commercial DNase II preparations have very high RNase activities. If the 14*S* fraction (fraction 10, figure 8) is exposed to 2 mM $MgCl_2$ and then rerun in an isokinetic gradient, the bulk of the nucleic acid sediments to the bottom of the centrifuge tube. However, about 50–70 % of the radioactivity resediments at 14*S*, in association with 5–10 % of the original input DNA. These data strongly suggest that nascent RNA chains are found in 14*S* subunits of $Mg^{2+}$-soluble chromatin.

## Fine structure of chromatin subunits

Noll (1974*b*) has shown that digestion of chromatin with pancreatic DNase I generates a regular series of single-stranded DNA fragments which are multimers of a ten nucleotide repeat. It is thought that this repeat pattern reflects the structure of DNA in the nucleosome cores (Noll 1974*b*; Crick & Klug 1975; Simpson & Whitlock 1976; Lutter 1977). Is the DNA of actively transcribed chromatin subunits organized in a similar manner? Figure 9 shows that upon digestion of 14*S* subunits with DNase I a typical ten-nucleotide repeat is generated. The major difference between DNase I digestion of 11*S* nucleosomes and 14*S* subunits is the rate of enzymatic attack. The digestion of 14*S* subunits with pancreatic DNase is much more rapid than the digestion of 11*S* nucleosomes. This is seen graphically in figure 10. The rate of digestion of $Mg^{2+}$-soluble chromatin DNA is essentially identical to the rate of digestion of deproteinized

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DNA. This finding is consistent with the results of Weintraub & Groudine (1976) and Garel & Axel (1976). These investigators report that active genes are preferentially attacked by DNase I. If nuclei are first digested with DNase I and then fractionated according to the DNase II/$Mg^{2+}$-solubility procedure, no material is recovered in the $Mg^{2+}$-soluble fraction. In fact, the first 20% of rat liver chromatin DNA digested by DNase I is the same 20% of DNA which is isolated in the $Mg^{2+}$-soluble fraction (figure 11).

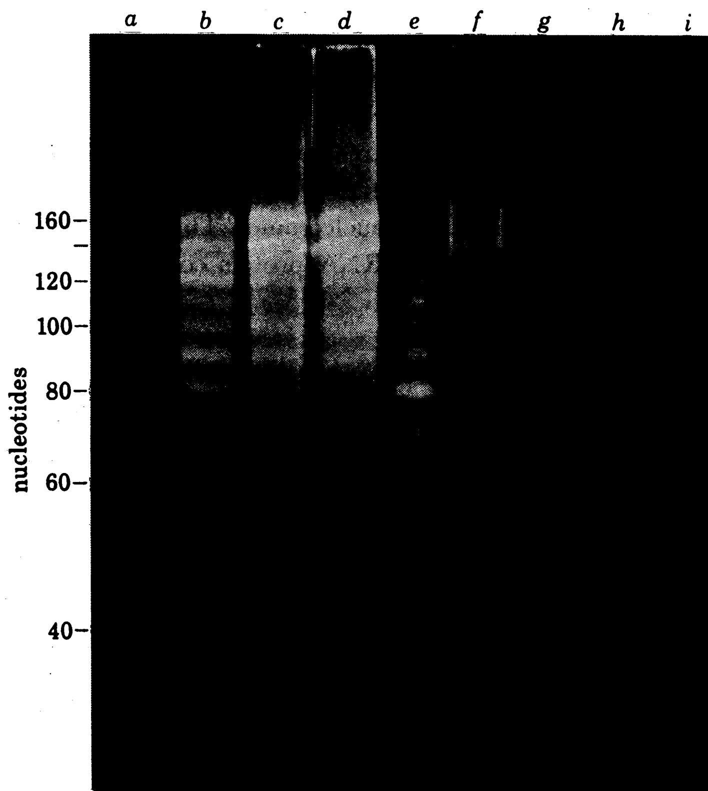


FIGURE 9. Electrophoresis of products of pancreatic DNase I digestion of chromatin monomers. Rat liver nuclei were treated with either DNase II (1 min at 2.5 units/$A_{260}$ unit) or with micrococcal nuclease (3 min at 300 units/ml) under standard conditions. The DNase II treated chromatin was fractionated as described. Total nuclear lysates of the micrococcal nuclease treated nuclei and the $Mg^{2+}$-soluble fraction of the DNase II treated chromatin were run on 5–24% isokinetic sucrose gradients. Chromatin monomers, recovered from the gradients, were digested with pancreatic DNase I at 30 units/ml in 10 mM $MgCl_2$ with 10 mM Tris–Cl (pH 8) at 37 °C. Reactions were stopped by pipetting 5 volumes of digest into 1 volume of 0.5% sodium dodecylsulphate in 0.1 M EDTA (pH 8). The DNA was purified and concentrated by ethanol precipitation. The DNA samples were dissolved in 50% formamide with 40% sucrose in standard gel buffer and denatured for 5 min in a boiling water bath. Electrophoresis was for 16 h at 35 mA in a 10% polyacrylamide slab gel containing 7 M urea. The gel was stained with ethidium bromide (1 µg/ml) and photographed. (*a*)–(*d*) Products of DNase I digestion of total chromatin monomer (produced by micrococcal nuclease digestion); (*e*) standard DNase I digest (300 units/ml for 30 s) of rat liver nuclei; (*f*)–(*i*) products of DNase I digestion of $Mg^{2+}$-soluble chromatin monomer. Digestion times: (*d*), (*f*), 30 s; (*c*), (*g*), 1 min; (*b*), (*h*), 2 min; (*a*), (*i*), 5 min.

## DNA CONFORMATION IN CHROMATIN SUBUNITS

Chromatin DNA is highly stabilized against thermal denaturation (Li & Bonner 1971). In 0.2 mM EDTA (pH 8), 11*S* nucleosomes exhibit a $T_m$ of 79 °C, with minor transitions at 68 and 84 °C (figure 12). In contrast, protein-free DNA melts at 42 °C. The 14*S* particles of $Mg^{2+}$-soluble chromatin exhibit an average $T_m$ of 55 °C, 24 °C below the $T_m$ of nucleosomal

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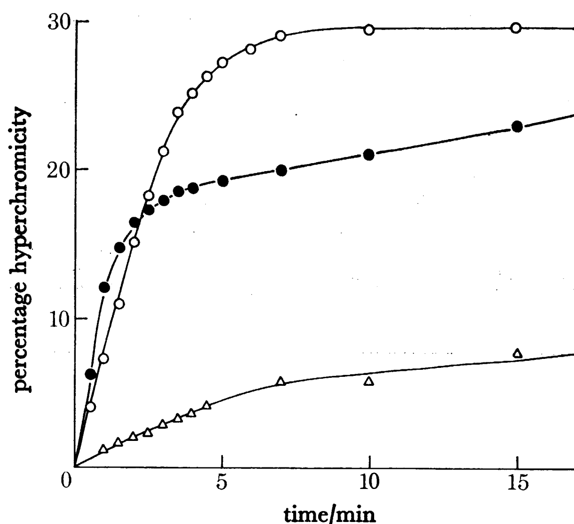


FIGURE 10. Kinetics of digestion of DNA and chromatin fractions with pancreatic DNase I. Rat liver chromatin was treated with DNase II (10 units/$A_{260}$ unit for 5 min) and fractionated as described. Chromatin fractions or purified DNA were digested with DNase I (100 units/ml, about 1 μg/ml) in 1 mM Tris–Cl (pH 8) containing 10 mM $MgCl_2$. The $A_{260}$ values of the substrate solutions were 0.5–0.7. The reaction was monitored spectrophotometrically at 260 nm. ○, DNA; ●, S2 chromatin; △, P1 chromatin.

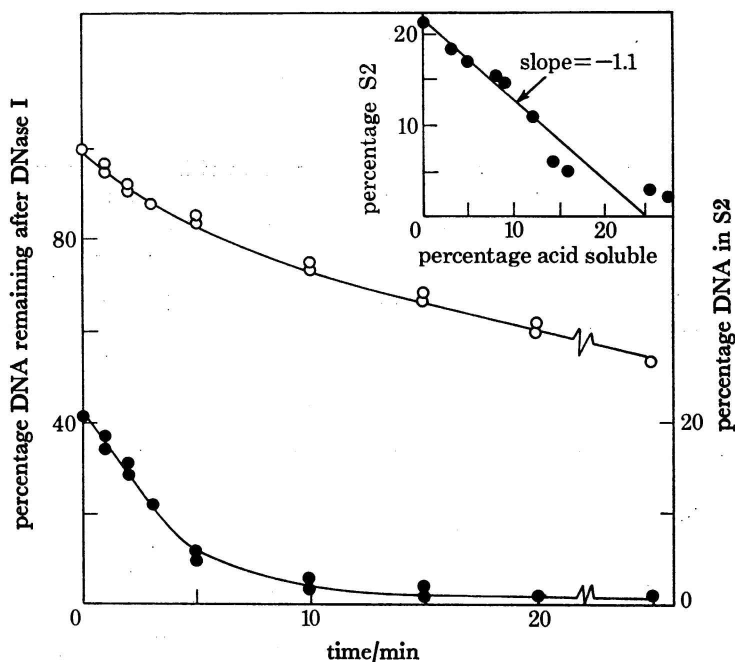


FIGURE 11. Effect of prior DNase I digestion of nuclei on recovery of $Mg^{2+}$-soluble (S2) chromatin. Rat liver nuclei were prepared and digested with DNase I (20 μg/ml) as described by Weintraub & Groudine (1976). At the times indicated, aliquots were withdrawn and pipetted into 5 volumes of 10 mM EDTA (pH 8). The nuclei were centrifuged and washed twice more in 0.2 mM EDTA, pH 8. The nuclei were lysed with a pasteur pipette (Noll *et al.* 1975). An aliquot of this sample was taken for a determination of the amount of DNA remaining after the initial DNase I digestion. The remaining chromatin was treated with DNase II and fractionated as described. ○, Percentage of original nuclear DNA remaining after DNase I digestion; ●, percentage of original nuclear DNA found in the $Mg^{2+}$-soluble (S2) chromatin fraction after subsequent DNase II treatment and fractionation.

Inset: percentage of DNA recovered in S2 chromatin plotted against percentage of DNA rendered acid soluble by initial DNase I digestion.

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DNA. Melting transitions at 42, 52, 66 and 77 °C can be recognized in a derivative plot of the data (figure 12). Deproteinized DNA and 11$S$ nucleosomal DNA exhibit total hyperchromicities of about 35 %. On the other hand, 14$S$ subunits exhibit a hyperchromicity of 11–20 % (range of observed values). Purified DNA from 14$S$ subunits exhibits a normal hyperchromicity and $T_m$. These data suggest that the DNA base pairs of 14$S$ subunits are partially unstacked (denatured) relative to protein-free DNA or 11$S$ nucleosomal DNA. Preliminary experiments indicate that the DNA of 14$S$ subunits is highly susceptible to attack by the single-strand-specific nuclease S1, while 11$S$ nucleosomal DNA is totally resistant to this enzyme.

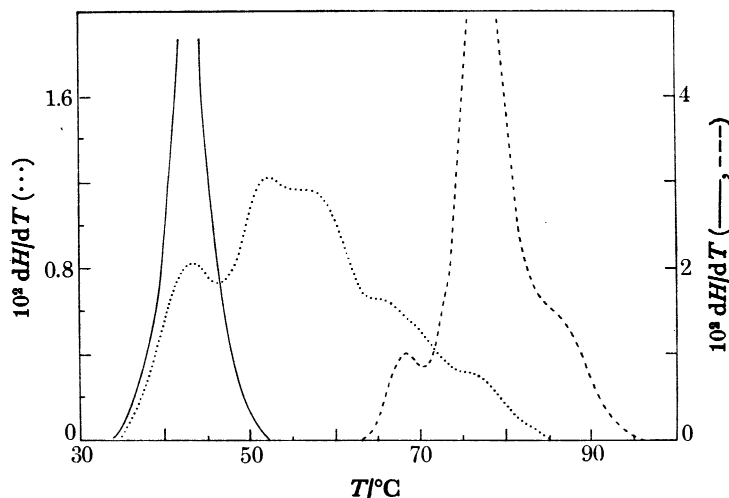


FIGURE 12. Derivative plots of optical thermal denaturation profiles of DNA and chromatin monomers. Rat liver chromatin was treated with DNase II for 5 min at 10 units/$A_{260}$ unit and fractionated as described. $Mg^{2+}$-soluble (S2) chromatin was centrifuged in a 5–24 % isokinetic sucrose gradient and the 14$S$ monomer was recovered from the gradient. Another sample of chromatin was treated with DNase II for 45 min, and the first supernatant fraction (S1) was centrifuged in an isokinetic sucrose gradient. 11$S$ monomers were recovered from the gradient. Chromatin monomers and purified rat DNA were dialysed against 0.2 mM EDTA (pH 8) before melting. ——, DNA; - - -, 11$S$ monomers; · · ·, 14$S$ monomers of S2 chromatin.

## CONCLUSION

Repeating nucleoprotein subunits occur in both transcribed and non-transcribed regions of chromatin. The amount of DNA and histone per subunit appears to be the same for these two classes of chromatin; however, transcriptionally active subunits are enriched in RNA and non-histone proteins. Thermal denaturation and enzyme digestion studies suggest that inactive nucleosomal DNA is tightly complexed with histone protein while the DNA of active chromatin is in a different, more open conformation. Further studies are needed to elucidate the conformational differences between active and inactive subunits. Alteration of nucleosome structure appears to be one of the steps leading to genetic activity (Weintraub, Worcel & Alberts 1976).

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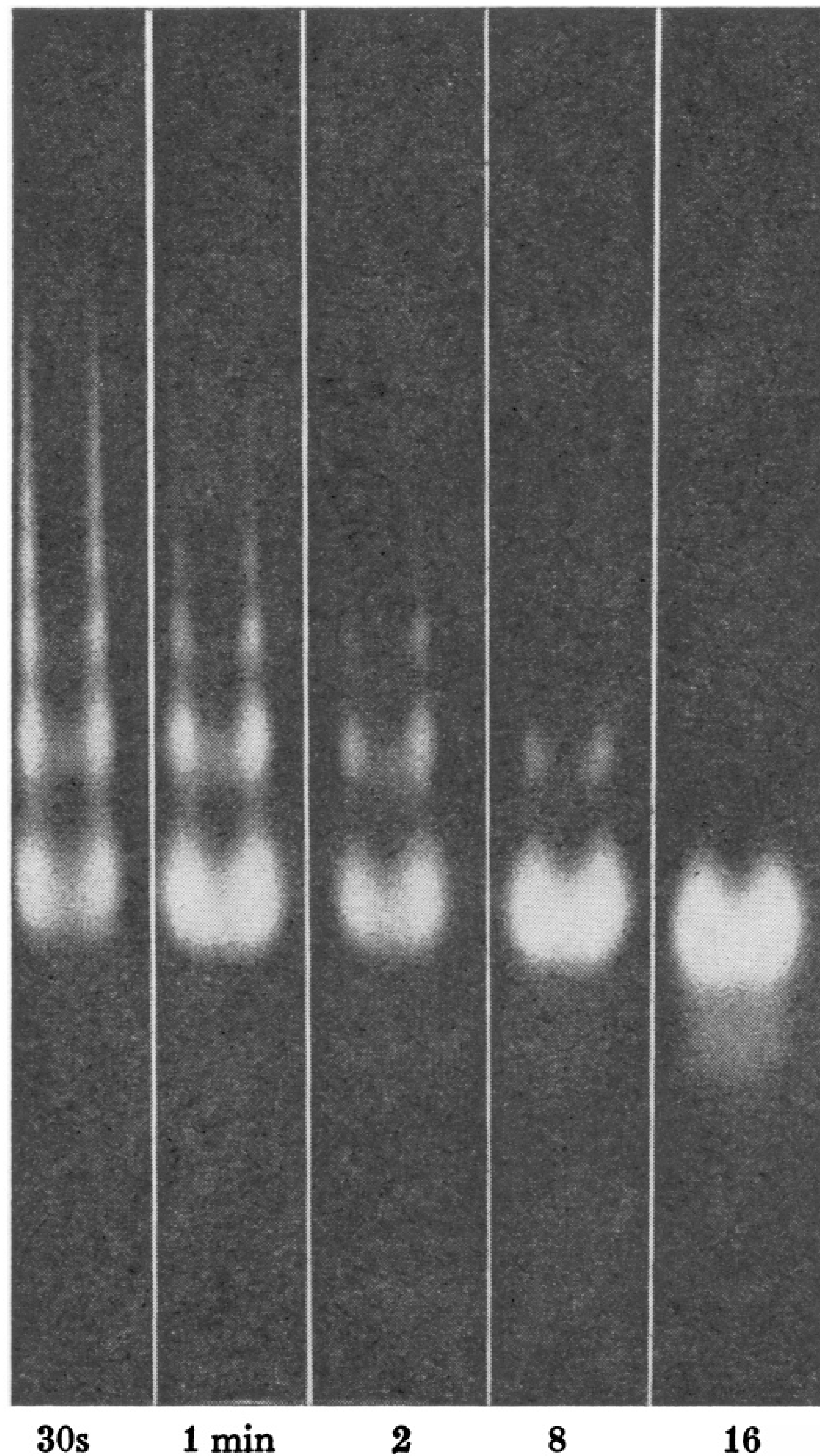


FIGURE 5. Agarose gel electrophoresis of $Mg^{2+}$-soluble chromatin DNA. Rat liver nuclei were lysed and treated with DNase II (4 units/$A_{260}$ unit) as described in the text. At each of the times indicated an aliquot of the chromatin suspension was withdrawn and fractionated. Purified DNA from fraction S2 (approximately 5–10 μg per sample) was electrophoresed for 14 h at 24 mA in a 2% agarose gel. After electrophoresis the gel was stained with ethidium bromide (0.5 μg/ml) and photographed.

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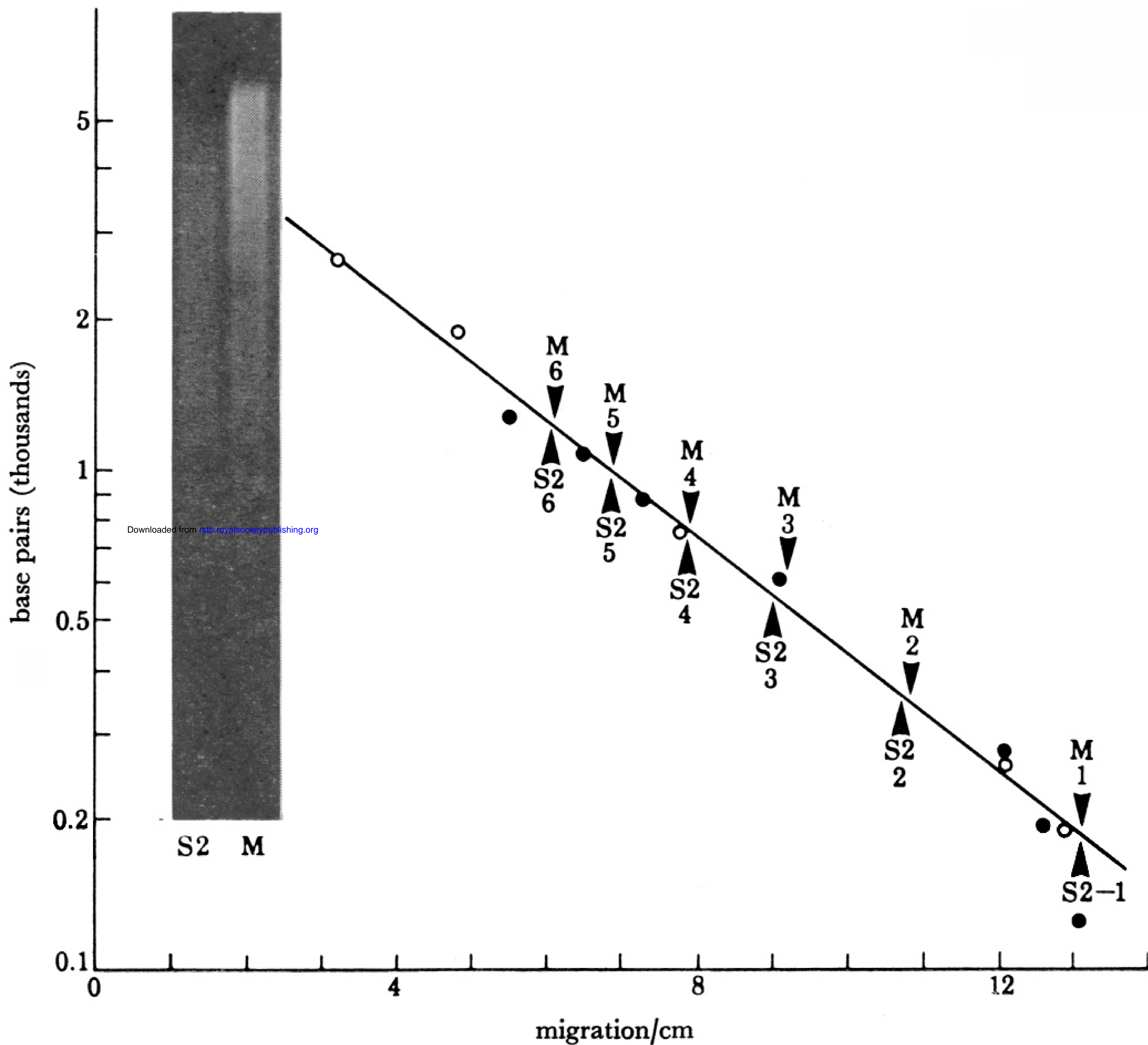


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FIGURE 6. Determination of the size of DNA fragments from nuclease digestions of chromatin. Rat liver nuclei were treated with DNase II (4 units/$A_{260}$ unit for 1 min) and the chromatin was fractionated as described. Nuclei were also treated with micrococcal nuclease (100 units/ml for 3 min at 37 °C) with no subsequent fractionation. DNA was purified from the micrococcal nuclease treated nuclei and from the $Mg^{2+}$-soluble fraction (S2) of the DNase II treated chromatin. Aliquots (10 µg) of each DNA sample were electrophoresed for 18 h at 30 mA in a 2% agarose gel. Sequenced restriction fragments (Hae II and Hae III) of ϕX 174 RF DNA were used for size determination. ○, Hae II fragments; ●, Hae III fragments: M1–M6, position of micrococcal nuclease generated chromatin DNA fragments, monomer to hexamer; S21 to S26, position of DNase II generated fragments of $Mg^{2+}$-soluble chromatin DNA, monomer to hexamer. Gel: S2, $Mg^{2+}$-soluble chromatin DNA produced by DNase II digestion; M, micrococcal nuclease digest.

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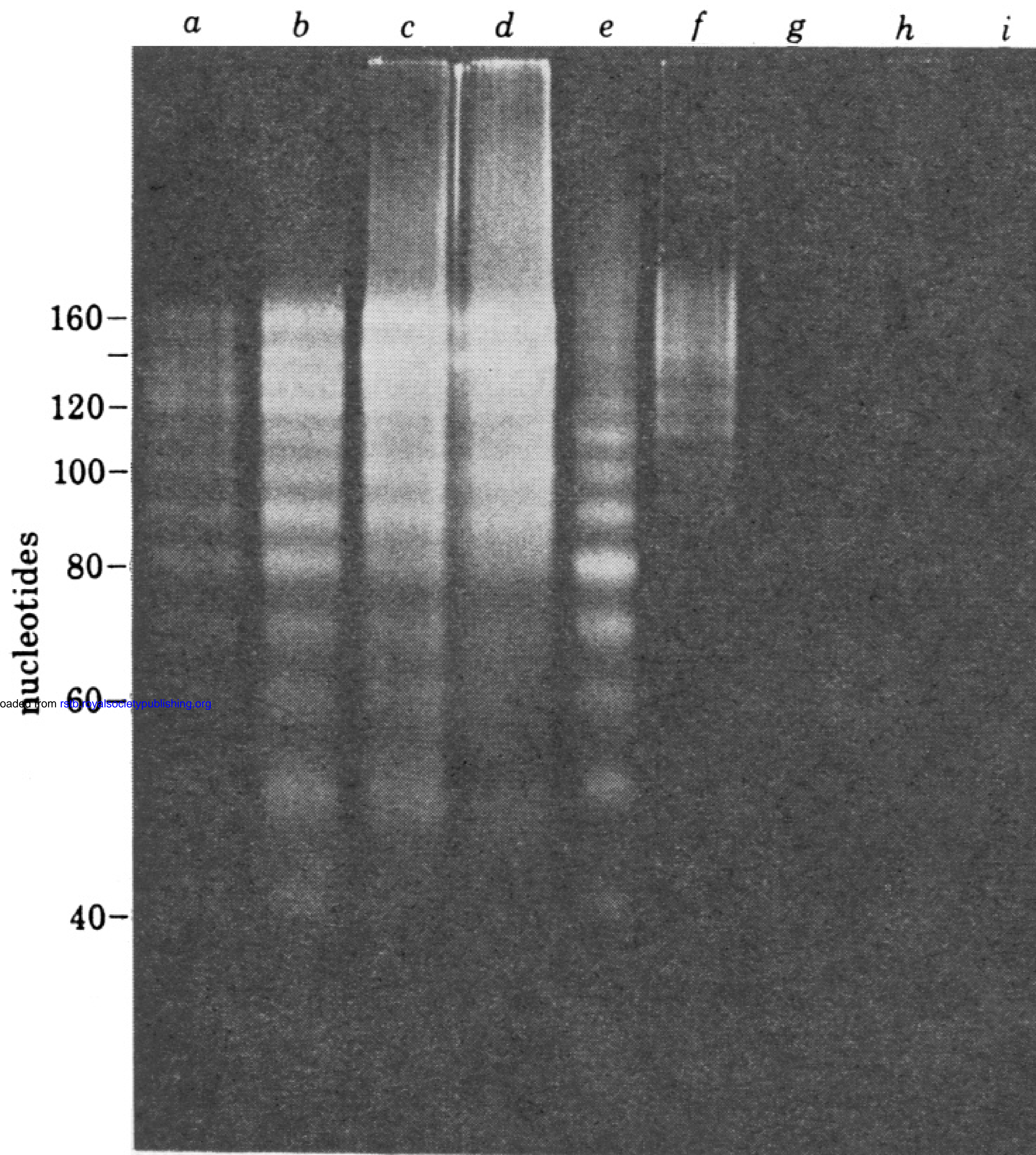


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FIGURE 9. Electrophoresis of products of pancreatic DNase I digestion of chromatin monomers. Rat liver nuclei were treated with either DNase II (1 min at 2.5 units/$A_{260}$ unit) or with micrococcal nuclease (3 min at 300 units/ml) under standard conditions. The DNase II treated chromatin was fractionated as described. Total nuclear lysates of the micrococcal nuclease treated nuclei and the $Mg^{2+}$-soluble fraction of the DNase II treated chromatin were run on 5–24 % isokinetic sucrose gradients. Chromatin monomers, recovered from the gradients, were digested with pancreatic DNase I at 30 units/ml in 10 mM $MgCl_2$ with 10 mM Tris–Cl (pH 8) at 37 °C. Reactions were stopped by pipetting 5 volumes of digest into 1 volume of 0.5 % sodium dodecylsulphate in 0.1 M EDTA (pH 8). The DNA was purified and concentrated by ethanol precipitation. The DNA samples were dissolved in 50 % formamide with 40 % sucrose in standard gel buffer and denatured for 5 min in a boiling water bath. Electrophoresis was for 16 h at 35 mA in a 10 % polyacrylamide slab gel containing 7 M urea. The gel was stained with ethidium bromide (1 μg/ml) and photographed. (*a*)–(*d*) Products of DNase I digestion of total chromatin monomer (produced by micrococcal nuclease digestion); (*e*) standard DNase I digest (300 units/ml for 30 s) of rat liver nuclei; (*f*)–(*i*) products of DNase I digestion of $Mg^{2+}$-soluble chromatin monomer. Digestion times: (*d*), (*f*), 30 s; (*c*), (*g*), 1 min; (*b*), (*h*), 2 min; (*a*), (*i*), 5 min.

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