Differential dissociation of chromatin digests: a novel approach revealing a hierarchy of DNA–protein interactions within chromatin domains

ANATOLY V. LICHTENSTEIN1,*, MIKHAIL M. ZABOIKIN1, NIKOLAI I. SJAKSTE2 and RAISA P. ALECHINA1

1Laboratory of Tumor Biochemistry, Institute of Carcinogenesis, All-Union Cancer Research Center, 24 Kashirskoye Shosse, Moscow 115478, USSR
2Latvian Institute of Experimental and Clinical Medical Research, Riga, Latvian SSR

*Author for correspondence

Summary

We describe here a novel approach to the dissection of chromatin structure by extracting DNA fragments from digested nuclei irreversibly immobilized (via proteins) on Celite columns. Three successive gradients (NaCl, LiCl–urea, temperature) are used to release three families of DNA fragments: namely, the 'DNA adherence' classes DNA-0, DNA-I and DNA-II, respectively. This 'protein image' DNA chromatography separates DNA fragments in accordance with the tightness of their bonds with proteins in situ. There are at least two DNA–skeleton attachment sites differing from each other by their resistance to the dissociating agents used as well as their susceptibility to DNAse I and S1 nuclease treatments, DNA cross-linking and single-stranded breaks. Several lines of evidence show a specific, topological rather than chemical, DNA–protein linkage at the tight attachment site. A hierarchy of chromatin loops demarcated by these attachment sites was determined. The technique described is generally applicable and can be used both to probe DNA–protein interactions and to map specific DNA sequences within the chromatin domain.

Key words: method, chromatin, nucleoskeleton, DNA fractionation.

Introduction

In eukaryotic cells linear DNA is organized into arrays of supercoiled loops tightly attached to a proteinaceous skeleton structure (reviewed by Agutter and Richardson, 1980; Newport and Forbes, 1987; Cook, 1988). It is generally assumed that the DNA sequences at the scaffold attachment sites are involved in such fundamental processes as replication and transcription (reviewed by Bodnar, 1988; Cook, 1988). Investigations aimed at understanding the composition of nuclear matrix proteins suggest that they vary in a cell-type-specific manner and may differ in normal and tumor cells (Kuzmina et al. 1984; Fey and Penman, 1988). A growing body of evidence indicates that nonrandom DNA sequences take part in anchoring DNA loops to the nuclear scaffold. The scaffold-attached regions were found to copart with functionally significant DNA sequences, such as replication origins (Goldberg et al. 1983; Razin et al. 1986; Marilley and Gassend-Bonnet, 1989), enhancer elements (Gasser and Laemmli, 1986), DNAse I-hypersensitive sites (Gross and Garrard, 1987), topoisomerase II consensus sequences (Greenstein, 1988), autonomously replicating sequences (ARSs) (Amati and Gasser, 1988; Sykes et al. 1988) and bent structures (Anderson, 1986).

The results presented here could be interpreted to show some heterogeneity of the sites where DNA and the nucleoskeleton interact. Bearing this in mind, we have examined the hierarchy of DNA–protein interactions within a chromatin domain using a novel approach of differential dissociation of chromatin digests, shown schematically in Fig. 1. The fact that adsorbent Celite irreversibly binds proteins, both free and complexed with nucleic acids, including complexes as large as polyribosomes or even isolated nuclei (Lichtenstein et al. 1982), makes it possible to treat immobilized nucleoproteins as natural chromatographic elements and elute DNA or RNA molecules from their protein environments in situ using various dissociating agents. Fractionation of nucleic acids as constituents of nucleoprotein complexes (nucleoprotein–Celite, or NPC chromatography) allowed us to separate efficiently the main classes of cellular nucleic acids (DNA, heterogeneous nuclear RNA, 28S and 18S ribosomal RNA) in a single chromatographic run (Lichtenstein et al. 1975, 1982) thus indicating the specificity of nucleic acid–protein interactions within the respective complexes. Apart from the ability to fractionate nucleic acids on the basis of a new principle, NPC chromatography has the potential for probing DNA–protein chemical bonds that stabilize a given complex by testing its sensitivity to various dissociating agents.

In this work, we used modified triple-gradient NPC chromatography to analyze chromatin digests from eukaryotic cells. In keeping with the anticipated high heterogeneity of DNA–protein interactions along the chromatin fiber we obtained a wide range of DNA fragments that were probably specific subdomain elements. The combined evidence strongly suggests the
Microdialysis chromatography and DNA analysis

Cells (0.5 × 10^6 to 1.0 × 10^6, i.e., 5–10 μg DNA) were lysed in a solution of 250 mM sucrose, 60 mM KCl, 15 mM NaCl, 3 mM MgCl₂, 15 mM Tris–HCl, pH 7.6, 0.5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride containing 0.1% diethyl pyrocarbonate was introduced into the lysing solution, the phosphate buffer (10 mM, pH7.6) being substituted cases 0.1% diethyl pyrocarbonate was introduced into the lysing solution, the phosphate buffer (10 mM, pH7.6) being substituted.

Chromatin digestion, NPC chromatography and DNA analysis

Cells were digested with 0.4–20 units per μg DNA at 2°C for 5 min; (2) restriction endonuclease BspRI (Ferment, USSR) – nuclei (0.5 × 10^6 to 1.0 × 10^6) were suspended in 0.5 ml of restriction buffer (50 mM NaCl, 25 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 10 mM 2-mercaptoethanol) and digested with the enzyme (up to 1000 units ml⁻¹) for 60 min at 37°C; the reaction was stopped by cooling and immediate adsorption on Celite; (3) S₁ nuclease (Biorad, USSR) – nuclei were used for the isolation of dehistonized DNA–matrix complexes (Berezney and Buchholz, 1981), which were suspended in 0.2 M NaCl, 20 mM sodium acetate, pH 4.5, 1 mM ZnSO₄ (0.4 mg DNA ml⁻¹) of incubation solution and digested with the enzyme (400 units ml⁻¹) for 60 min at 37°C; the reaction was stopped by cooling and the sample was immediately added to the Celite suspension; (4) Bal31 nuclease (Ferment, USSR) – DNA–matrix complexes isolated as in (3) were suspended in 0.5 M NaCl, 2 mM Tris–HCl, pH 8.1, 12.5 mM MgCl₂, 12.5 mM CaCl₂, 1 mM EDTA, and digested with the enzyme (500 units ml⁻¹) for 60 min at 30°C.

Differential dissociation of chromatin digests was performed either by the standard triple-gradient procedure (outlined below) or by the double-gradient method (Lichtenstein et al., 1982), omitting the first NaCl gradient (the latter procedure was used to analyze dehistonized DNA–matrix complexes). Isolated nuclei (0.5 × 10^6 to 1.0 × 10^6) were added into previously prepared column they are adsorbed in a narrow upper Celite layer, thus giving rise to subsequent intermolecular interactions between released DNA strands, resulting in column obliteration and poor resolution. Subsequent fractionation of DNA fragments and their analysis are shown schematically in Fig. 1. Both the eluents used (NaCl, LiCl–urea) were prepared with TM buffer. The family of fragments designated DNA-0 (fractions 2, 3, 4) was eluted with 90 ml of NaCl gradient (0 to 3 M) at 2°C, the ‘washing-through’ fraction being no 1. The fragment family DNA-I was eluted with 60 ml of LiCl–urea gradient (from 0 to 4 M and 8 M, respectively) at 2°C. The fragment family DNA-II was eluted with approximately 60 ml of 4 M LiCl–8 M urea during temperature elevation from 2°C to above 95°C, the DNA 100 ml being in the range from 2°C to 70°C, while the last was within the 70 to 95°C interval. The eluates were collected either in 70–80 fractions (or less, if the double-gradient procedure was used) of 3 ml with high resolution was needed, or in 8 fractions of 30 ml when the DNA was to be used for further analysis (electrophoresis, hybridization). In the latter case, the fractions of a given family are indicated by subscripts, e.g. DNA-0₁, DNA-1₂, DNA-1₁.

The relative content of DNA in the fractions was determined either by in vivo labeling with radioactive (³²P)- or (¹⁴C)-thymidine (0.1–0.5 μCi ml⁻¹) as described in the text or by DNA-Hoechst 33258 fluorescence in a Perkin-Elmer 3000 spectrometer. In the latter case, DNA in 30 ml fractions was adsorbed onto hydroxyapatite (HAP) (Bio-Rad, Richmond, CA) as described earlier (Lichtenstein et al., 1989) and washed with 0.01 M sodium phosphate buffer, pH 6.8. HAP was dissolved overnight, with gentle shaking in 600 μl of 0.5 M EDTA, pH 8.0, at 37°C. The solution was then clarified by centrifugation at 1000 g for 5 min, the supernatant saved and DNA, after dialysis and digestion with RNase A (50 μg ml⁻¹, 1 h, 37°C) and with 50 μg ml⁻¹ of proteinase K in 10 mM EDTA, 1% Scrosol at 60°C for 30 min, was precipitated with isopropanol, washed with 70% ethanol and subjected to analysis. Electrophoresis in a horizontal 1% agarose gel in the presence of 89 mM Tris–phosphate, 5 mM EDTA, pH 8.0, buffer was carried out as described (Maniatis et al., 1982).

Materials and methods

Cell growth and labeling

Growth conditions and labeling of HeLa cells were essentially as described earlier (Lichtenstein et al. 1982) and also described in detail in the text.

Chromatin digestion, NPC chromatography and DNA analysis

Cells (0.5 × 10^6 to 1.0 × 10^6, i.e., 5–10 μg DNA) were lysed in a solution of 250 mM sucrose, 60 mM KCl, 15 mM NaCl, 3 mM MgCl₂, 15 mM Tris–HCl, pH 7.6, 0.5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride containing 2% Triton X-100. In some cases 0.1% diethyl pyrocarbonate was introduced into the lysing solution, the phosphate buffer (10 mM, pH7.6) being substituted.

Break-through fraction

NaCl, 2°C

LiCl–urea, 2°C

Temperature

(2–96°C)

30 ml fractions

DNA-0

DNA-I

DNA-II

Hydroxyapatite adsorption

Dissolution in 0.5 M EDTA microdialysis electrophoresis

Treatment with 0.5 M NaOH (60°C, 1 h) to remove RNA, dissolution in 25% TCA, transfer DNA to membrane filter for dot hybridization with DNA probes

Dissolution in 10% HClO₄ and [³²P]DNA counting in Triton X-100-toluene mixture

Fig. 1. General scheme of differential dissociation of chromatin digests. Isolated nuclei or DNA–matrix complexes are immobilized on Celite by irreversible binding of proteins. Nucleic acids are released by successive gradients of NaCl, LiCl–urea and temperature. By means of adsorption on HAP, DNA is concentrated and subjected to further analysis.

existence in the nucleus of two types of DNA–skeleton interaction that can be distinguished by the structural criteria as well as by some functional properties.
mmol⁻¹) for 2 days were digested with BspRI (0.1–1000 units ml⁻¹, 1 h at 37°C) and subjected to NPC chromatography. DNA fractions treated as described above were size-fractionated in a 1% agarose (LMP grade, BRL) gel and stained with ethidium bromide (0.5 μg ml⁻¹). After photography, gel tracks were sliced and the slices (4 mm) were dissolved in 2 ml of 100 mM sodium acetate (pH 5), 1 mM EDTA, 1% SDS and 0.5% sodium dodecyl sulphate and counted. The experimental data were used to obtain the curve according to the expression:

\[ \text{D}(x(n)) = \sum_{i=1}^{n} \text{cts} \min^{-1}(i) \bigg/ \sum_{i=1}^{m} \text{cts} \min^{-1}(i) \bigg, \]

where \( x(n) \) is the size (in bp) of a molecule at the lower edge of the slice, \( \text{cts} \min^{-1}(i) \) is the radioactivity of the slice \( i (i=1 \text{ to } m) \) is the total slice number. The \( \text{D}(x(n)) \) curves obtained were treated in two ways. First, weight average molecular weights, number average molecular weights and loops sizes were calculated according to the best fit approach of Jackson et al. (1990). The second approach is based on theoretical descriptions given by Tanford (1966) for linear polymers. The \( \text{D}(x(n)) \) curve obtained was approximated by the theoretical expression \( \text{F}(x,a) = (1 - x \ln(1 - 1/a))/(1 - 1/a)^2 \), where \( x \) is the size (in bp) of a molecule, and \( a \) is the number average molecular weight. The latter method, which is equivalent to the first one, upon substitution of \( \ln(1 - 1/a)=-1/a \), was chosen for further analysis throughout this work because of its compatibility with the computer program Eureka v.1.0 (Borland International, Inc.), which has performed a least squares fit to find the function that best matches the points. At this stage in the analysis both approaches gave similar results. Further refinement was performed as follows. The best fit was achieved upon presentation of \( \text{D}(x(n)) \) curves as consisting of two \( \text{F}(x,a) \) components. As a result, the maximal absolute error was decreased by an order of magnitude and became less than 0.06 for the \( \text{D}(x(n)) = \{ F(x,a_1) + (p \times F(x,a_2)/2,1-p)) \) function, where \( p \) is a portion of the \( F(x,a_1) \) component. The major component (more than 70% of the population) comprises large molecules (\( a_1 \) from 2 to 6 kbp), while much smaller molecules form a minor one (\( a_2 \) from 190 to 400 bp). Loop size determinations were carried out for the major component.

The extent of DNA fragmentation was estimated by the distribution of radioactivity in linear alkaline sucrose gradients as described earlier (Sjakste et al. 1983).

DNA crosslinking was performed by treatment of isolated DNA–matrix complexes suspended in TM buffer with 5 mM 8-methoxypsoralen for 10 min at 4°C and long-wavelength u.v. light from a CBD-120A tube (the peak irradiation wavelength was 360 nm). This procedure resulted in the formation of one crosslink per 1–2 kilobases (kb) on average, as tested by alkaline sucrose density gradient analysis.

Results

Chromatographic separation of chromatin digests

Fig. 1 is a schematic diagram of all subsequent experiments. Briefly, intact or nuclease-digested nuclei (0.5×10⁶ to 1.0×10⁶) or their derivatives (nucleoskeleton structures in association with DNA) are immobilized on Celite columns and subjected to the differential DNA–protein dissociation procedure, which consists of three successive gradients (NaCl, LiCl–urea and temperature) to release three families of DNA fragments (DNA-0, DNA-I and DNA-II, respectively). Small amounts of DNA samples available for analysis (5–10 μg, total) highly diluted with eluent solutions (more than 200 ml, total) require special methods of DNA concentration to be elaborated (see Materials and methods).

Fig. 2 shows the chromatographic patterns of DNA from isolated HeLa nuclei, both intact and gradually digested with DNase I. Exponentially growing HeLa cells were labeled with [3H]thymidine (1 μCi ml⁻¹) for 24 h. Isolated nuclei were subjected to graded DNase I digestion followed by triple-gradient NPC chromatography. (A) Intact cells; (B,C,D) nuclei with 1.9, 7.4 and 86% DNA hydrolyzed, respectively.
most cells studied (0.4 Kunitz units μg⁻¹ DNA, 5 min at 2°C) are possibly within the range of conditions usually used for the determination of DNAase I-sensitive and hypersensitive chromatin regions.

One more circumstance is noteworthy. As will be discussed below in more detail, the borderline between DNA-0 and DNA-I marked by the end of the NaCl gradient is operationally considered by us as a boundary between the DNA fragments that are attached and those that are not attached to the nucleoskeleton. This assumption is in agreement with the widely adopted arbitrary recognition of the nucleoskeleton (scaffold, matrix) as the nuclear remnants that resist treatment with solutions of high ionic strength (usually 2 M NaCl). Hence, it follows that, although at the early stages of DNAase I digestion most of the DNA remains bound to the nucleoskeleton (Fig. 2B), this complex is no longer native as demonstrated by the disappearance of the tight DNA-II attachment site. Apparently, this DNAase I-induced transition can pass unnoticed during standard experiments dealing with total (nonfractionated) skeleton-attached DNA fragments.

DNA fragment size is not a factor of fractionation

The above-mentioned tight association of intact DNA with the nucleoskeleton may be attributed to the artefactual 'trapping' of giant DNA strands within the intranuclear fibrillar network. Decrease in the size of DNA as a result of digestion should reduce such an effect and facilitate the release of DNA fragments from the nucleus. Thus, the results shown in Fig. 2 can be explained by the elimination of the artefactual 'trapping' effect. To check the suggestion we compared by gel electrophoresis the sizes of DNA fractions obtained from HeLa nuclei, both intact (Fig. 3A) and digested with either restriction endonuclease BspRI (B, C) or DNAase I (D).

Intact HeLa nuclei (Fig. 3A) are seen to contain tightly bound DNA-II and DNA-II₂ as major fractions that consist of large molecules (more than 24 kbp). It should be stressed that this size value is apparently highly underestimated, since an inevitable DNA shearing should take place from the moment of DNA release from nuclei and later on during all the post-chromatography procedures such as HAP adsorption and multiple DNA pipetting. So, the DNA molecules just released from nuclei would probably be much larger. Besides DNA-II, some additional minor DNA fractions, also of large size but bound to the nuclear interior less tightly than DNA-II, are revealed (see, for example, DNA-II₁ fraction). This phenomenon of DNA adherence heterogeneity that is seen even in intact nuclei and initially described in our earlier report (Lichtenstein et al. 1982) is under investigation. Without describing the details of this phenomenon it is appropriate to note that the minor fractions mentioned, which can be easily overlooked upon standard fractionation (see Fig. 2), usually manifest themselves only after concentration from initially highly diluted solutions.

Fig. 3B,C shows a sharp decrease in DNA fragment size concomitant with a DNA shift to the left as a result of digestion of nuclei with BspRI. The relative amount of easily extractable DNA-0₁₂ fractions increases as digestion proceeds. Qualitatively the same changes, but expressed even more significantly, are revealed upon nuclear digestion with DNAase I (Fig. 3D). In the latter case, a significantly decreased amount of material is recovered in the NPC fractions because of the ability of DNAase I to digest DNA to acid-soluble products. The marked overlapping of DNA sizes that is seen on inspection of DNA fractions belonging to various adherence classes (from nuclei both intact and digested) indicates that DNA size is not a factor in fractionation. On the other hand, a small portion of tightly bound DNA-II₁ and DNA-II₂ fractions resists digestion and remains attached to the nucleoskeleton despite a marked decrease in size (see Fig. 3B, C, and D). As will be shown below, DNA fragmentation weakens the tight type II attachment, which is presumably the reason why a predominant portion of tightly bound fragments is usually found in the DNA-II₁ subfraction rather than in DNA-II₂.

Quantitation of the amount of material in various DNA

Fig. 3. Size determination of DNA fractions separated by NPC chromatography. HeLa nuclei, either intact (A) or digested with BspRI (B and C, 100 and 500 units ml⁻¹, respectively, for 1 h at 37°C) or DNase I (D, 12 units ml⁻¹ for 5 min at 2°C), were used as starting material for triple-gradient NPC chromatography. DNA samples were concentrated and size-fractionated by agarose gel electrophoresis. Markers (in kb) are indicated on the right.

506 A. V. Lichtenstein et al.
digestion is the washing of treated nuclei, prior to preserved despite their small size association with the consistent finding of type I and type II fragments tightness (resistance to dissociation) of DNA-protein interactions that determines the chromatographic position as revealed by DNA staining with Hoechst 33258. Redistribution of material leads at the earliest stage of DNA digestion (1–2.5 units ml\(^{-1}\)) to a transient increase in DNA-II(1) and later on in DNA-I(1) and DNA-II(2) fractions. Finally, most detached DNA resides in DNA-0(2) and DNA-0(3) eluted from immobilized nuclei by NaCl gradient. This movement implies some hierarchy of DNA–protein attachment sites within chromatin domains.

Fig. 5 shows the results of size-fractionation of DNA fractions obtained after extensive chromatin digestion (500 units ml\(^{-1}\), 1 h at 37°C). In all cases DNA appears to be heterogeneous ranging in size from mononucleosomes to fragments 20 kbp long (except for the ‘washing-through’ DNA-0(1) and the most tenaciously bound DNA-II(2)). A clear-cut nucleosomal ladder seen in all DNA-0 fractions becomes less discernible (though easily visible on the original negative) in both DNA-I subfractions and DNA-II(2) fractions. Finally, most detached DNA resides in DNA-0(2) and DNA-0(3) eluted from immobilized nuclei by NaCl gradient. This movement implies some hierarchy of DNA–protein attachment sites within chromatin domains.

Table 2 summarizes the results of loop size determinations. It is appropriate to note here that the term loop size used later on for the sake of simplicity, can be attributed, strictly speaking, only to the tight attachment sites, while in all other occasions, because of the uncertainty of spatial interrelationships between various attachment sites, it means instead the size of the DNA stretch between two adjacent attachment sites of a specific type (see also Discussion). Bearing this in mind, we determined the loop sizes for DNA-II(2), DNA-II(1), DNA-

### Table 1. Effect of nuclease treatments on distribution of uniformly labeled DNA (in cts min\(^{-1}\))

<table>
<thead>
<tr>
<th></th>
<th>DNA-0</th>
<th>DNA-I</th>
<th>DNA-II</th>
<th></th>
<th>DNA-0</th>
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<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
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<td>Control</td>
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<td>6200</td>
<td>42800</td>
<td>32000</td>
<td>92400</td>
<td>49600</td>
<td>133,826</td>
</tr>
<tr>
<td></td>
<td>0.2 %</td>
<td>0.9 %</td>
<td>6.2 %</td>
<td>4.6 %</td>
<td>15.3 %</td>
<td>7.2 %</td>
<td>19.3 %</td>
</tr>
<tr>
<td>BspRI</td>
<td>18480</td>
<td>46200</td>
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<td>149820</td>
<td>15666</td>
<td>28974</td>
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<td></td>
<td>2.7 %</td>
<td>6.7 %</td>
<td>42.4 %</td>
<td>21.6 %</td>
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<td>137841</td>
<td>13629</td>
<td>21450</td>
<td>18054</td>
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<tr>
<td>BspRI</td>
<td>3.4 %</td>
<td>7.7 %</td>
<td>34.8 %</td>
<td>19.9 %</td>
<td>1.8 %</td>
<td>3.1 %</td>
<td>2.6 %</td>
</tr>
<tr>
<td>DNase I</td>
<td>16962</td>
<td>70389</td>
<td>137676</td>
<td>26466</td>
<td>4422</td>
<td>2706</td>
<td>4323</td>
</tr>
<tr>
<td></td>
<td>2.5 %</td>
<td>10.2 %</td>
<td>19.9 %</td>
<td>3.8 %</td>
<td>0.6 %</td>
<td>0.4 %</td>
<td>0.6 %</td>
</tr>
</tbody>
</table>

HeLa cells were labeled with \(^{3}H\)thymidine (0.25 µCi ml\(^{-1}\) for 1 day, isolated nuclei were treated as indicated, washed twice with TM buffer and subjected to NPC chromatography.

populations was carried out by NPC analysis of nuclease digests of HeLa nuclei uniformly labeled with \(^{3}H\)thymidine. The results of a representative experiment presented in Table 1 corroborate those shown above: namely, the shift of the bulk of DNA from type II attachment to the left leaving behind a fraction as small as 0.1 % (in the case of DNAase I digestion). The reason for a notable loss (15–25 %) in material recovered in NPC fractions after BspRI digestion is the washing of treated nuclei, prior to the analysis, with TM buffer, which can remove some easily extractable fractions. In the case of DNAase I treatment, the considerably larger loss of material than in the previous case (62 %) is explained by digestion of DNA to acid-soluble products.

Thus, the combined data show that neither the molecular size nor the trapping effect determines the separation by NPC chromatography. Rather, it is the tightness (resistance to dissociation) of DNA–protein interactions that determines the chromatographic positions of the nucleic acids studied. Besides this, a consistent finding of type I and type II fragments preserved despite their small size association with the nucleoskeleton, is evidence for the existence of at least two types of these complexes. Using a different method of nuclear immobilization (encapsulation in agarose beads), Jackson and Cook (1985) demonstrated the association of DNA with the nucleoskeleton at physiological ionic strength. The data in Figs 2 and 3 support this main conclusion and supplement it by demonstrating the heterogeneity of such interactions.

It was assumed above that DNA-0 originates from free (detached from the matrix) chromatin whereas DNA-I and DNA-II still preserve their association (weak and tight, respectively). This assumption was investigated by separate NPC analyses of various subnuclear fractions, such as free nucleosomes obtained by micrococcal digestion of HeLa nuclei and DNA–matrix complexes obtained by the method of Berezney and Buchholtz (1981). The results obtained (not shown here) support the notion that DNA-0 originates from free chromatin whereas the origin of DNA-I and DNA-II is from chromatin attached to the nucleoskeleton by weak and tight interactions, respectively.

### Determination of loop size

Isolated HeLa cell nuclei digested with 4 bp-cutter BspRI were subjected to chromatography and the DNA fractions obtained were size-fractionated, sliced and counted essentially as described by Jackson et al. (1990). Then loop size (or, more exactly, a distance between two adjacent attachment points characteristic of a DNA fraction given) was calculated from the percentage of DNA remaining in the fraction and the size of corresponding fragments.

Treatment of nuclei with increasing quantities of BspRI progressively removes DNA from the most tight attachment as revealed by DNA staining with Hoechst 33258 (Fig. 4). Redistribution of material leads at the earliest stage of DNA digestion (1–2.5 units ml\(^{-1}\)) to a transient increase in DNA-II(1) and later on in DNA-I(1) and DNA-II(2) fractions. Finally, most detached DNA resides in DNA-0(2) and DNA-0(3) eluted from immobilized nuclei by NaCl gradient. This movement implies some hierarchy of DNA–protein attachment sites within chromatin domains.

Subdomain chromatin structure

507
Fig. 4. HeLa nuclei digestion with BspRI restriction enzyme. Isolated nuclei were gradually digested and then subjected to chromatography. DNA fractions obtained were concentrated and measured by fluorescence with Hoechst 33258. Graphs show the percentage of DNA in chromatographic fractions after digestion with different amounts of the enzyme. Symbols for each fraction are under the figure.

Fig. 5. Estimation of loop size by nuclease digestion. 14C-labeled cells were lysed, incubated with BspRI (500 units ml⁻¹, 37°C, 1 h) and chromatographed. DNA fractions were concentrated, treated with proteinase K and size-fractionated. The photograph of gel illustrates the range of fragments in various DNA fractions. m, λ/HindIII–EcoRI digest.

Characterization of DNA–protein bonds

Elution of DNA-0 and DNA-I by, respectively, NaCl and LiCl–urea gradients indicates that the former is retained within the nucleus predominantly by ionic bonds whereas the latter is retained by ionic, hydrogen and, probably, hydrophobic bonds. Some reports have shown that disruption of the weak DNA–matrix association requires urea treatment. An important role for polar as well as hydrophobic interactions in combination with hydrogen bonds in maintaining the integrity of DNA–matrix contacts was suggested by Mullenders et al. (1982).

As for the DNA-II fraction, its dependence on heating for release from nuclei could imply the necessity for some melting of DNA. This is all the more probable as a preliminary experiment showed melting of purified eukaryotic DNA in a solution of 4 M LiCl–8 M urea in TM buffer upon heating above 75°C as revealed by the hyperchromic effect.

The suggestion that denaturation is a prerequisite for the liberation of DNA from its tight association with the nucleoskeleton was investigated in a study of the effects of factors that facilitate denaturation (low ionic strength) or hamper it (DNA crosslinking) on DNA-II chromatographic patterns. In the first case (i.e. with the substitution of 0.12 M sodium phosphate, pH 7.6, 8 M urea for the standard 4 M LiCl–8 M urea, TM buffer as the eluate) DNA-II is eluted considerably earlier and in a wider temperature range.

DNA-0(2) — DNA-0(3) — DNA-I(1) — DNA-II(1) — DNA-II(2)
range than usual (Fig. 6A), though the effect would have been the opposite if the complex studied had been stabilized only by ionic and hydrogen bonds. In contrast, the formation of 8-methoxypsoralen-induced DNA crosslinking leads to a more hampered release of the tightly bound DNA from isolated DNA–matrix complexes (Fig. 6B). Moreover, a sizeable portion of the crosslinked DNA appears irreversibly bound to the column (not shown). It is interesting to note that interstrand crosslinking produces no visible effect on the chromatographic pattern of DNA eluted with LiCl–urea gradient.

Taken together, these results imply that the DNA loop demarcated by the tight DNA-II–matrix anchorage sites remains bound to the matrix until the DNA is denatured. The weak DNA–matrix attachment is organized in some other way. It is our opinion that the most plausible organization of the weak and tight DNA–skeleton attachment would be that outlined in Fig. 7. The weak complex is formed by chemical (hydrogen, ionic, hydrophobic) interactions between DNA and the protein complex positioned outside the double helix. As for the tight DNA–matrix attachment site, it is stabilized by topological, rather than purely chemical, linkage between DNA and the protein complex protruding between two DNA strands in a locally denatured region—a 'press-stud' method of attachment as shown in Fig. 7. The nucleoskeleton–matrix 'stud' retains to the DNA until the two strands are separated. All the agents that favor DNA denaturation must facilitate DNA release from the tight attachment and, on the other hand, the agents stabilizing DNA helix, such as cross-linking, must hamper it (see Fig. 6A and B). Additionally, this hypothesis predicts a high sensitivity of type II interactions to single-strand-specific nucleases, since the attachment site is presumably locally denatured. Indeed, an important role for single-stranded regions in maintaining the integrity of the tight attachment site is suggested by digestion of DNA–matrix complexes with single-strand-specific nucleases Bal31 and S1 (Fig. 8), which induce drastic shifts of the DNA-II fraction to the left. In this context it is worth remembering the high DNAase I sensitivity of type II interactions noted above, which can also be explained by involving single-stranded regions in type II interactions. Apparently, a single-strand break in the sensitive site can be sufficient for the breakdown of type II attachment to occur.

<table>
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<th>Subdomain chromatin structure</th>
<th>509</th>
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Table 2. Loop size determinations

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>DNA-I</th>
<th>DNA-II</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>%DNA in fraction</td>
<td>3.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Number average mol. weight (kbp)</td>
<td>0.28</td>
<td>0.72</td>
</tr>
<tr>
<td>Estimated loop size (kbp)</td>
<td>126</td>
<td>118</td>
</tr>
</tbody>
</table>

HeLa cells were labeled with [14C]thymidine, isolated nuclei were treated as indicated, subjected to NPC chromatography and then the DNA fractions obtained were size-fractionated for loop size determination (see Materials and methods). %DNA in the most tightly bound fraction was calculated, excluding a satellite at the top of the gel. Proceeding from two-component kinetics of DNA cleavage in isolated nuclei by BspRI, percentages of all DNA-I and DNA-II fractions were determined for the major component (see the text).

* Loop size for total (nondiscriminated) attachment sites was deduced by summarizing all the types of attachment site revealed.

Fig. 6. Influence of the factors affecting DNA melting on NPC chromatographic patterns. (A) Effect of low ionic strength. Continuously growing HeLa cells were labeled with [14C]thymidine (0.5 μCi/ml) for 24 h and used for isolation of DNA–matrix complexes. The salt–urea gradient (not shown) was carried out as usual, while the temperature gradient was performed using 0.12 M sodium phosphate buffer, pH 7.6, instead of 4 M LiCl (TM buffer was omitted in this experiment). The other sample of DNA–matrix complexes was analyzed as usual ( ). The other sample of DNA–matrix complexes was analyzed as usual ( ). (B) Effect of DNA crosslinking. DNA–matrix complexes were isolated without DNase I treatment from continuously growing HeLa cells labeled with [3H]thymidine (0.5 μCi/ml) for 24 h. One portion was used as control ( ) and the other was crosslinked with 8-methoxypsoralen ( ). DNA–matrix complexes were subjected to the double-gradient procedure. Arrows indicate the start ( ) and progression of the temperature gradient.
DNA-I attachment site (hydrogen and ionic bonds)

DNA II-Scaffold topological linkage

Nucleosomes (ionic bonds)

Fig. 7. The model for chromatin domain organization. The model takes into account the occurrence of two DNA-matrix attachment sites. The DNA I-matrix attachment is stabilized by ionic and hydrogen bonds between DNA and the protein complex located outside the helix. The DNA II-matrix attachment is a topological linkage formed by locally separated DNA strands with a protein complex in between (the press-stud attachment).

Effect of single- and double-stranded breaks on DNA-II position

NPC chromatography is thought to be somewhat similar to the methods of alkaline or neutral elution of DNA from membrane-immobilized cell lysates (Kohn et al. 1976; Bradley and Kohn, 1979), which are widely used for quantitation of DNA damage. It is generally agreed that it is the size of DNA fragments, either double-stranded (ds) or single-stranded (ss), that determines the rate of neutral or alkaline elution, respectively, of DNA from membrane filters. Recently, however, an idea was put forward suggesting that the association of DNA with the nucleoskeleton may play an important role in the process of DNA elution and that ds breaks facilitate DNA release by eliminating this link rather than by reducing DNA fragment size (Russo et al. 1987). This reasoning is essentially similar to that concerning DNA chromatographic behavior upon nuclease digestion (see above).

We believe that NPC chromatography combines both approaches, neutral and alkaline elution, in a single chromatographic run by successively using agents with and without DNA denaturing capabilities: temperature and salt-urea gradients, respectively. The model for chromatin domain organization shown in Fig. 7 allows us to suggest a unified explanation as to why both ds and ss breaks facilitate DNA release by both methods, that of Kohn and collaborators and ours. Evidently, ds breaks facilitate DNA release by detachment of DNA fragments from the nucleoskeleton while ss breaks can do the same just after DNA denaturation. Besides, it is reasonable to suggest that ss breaks weaken the type II attachment itself by promoting DNA unwinding, the latter being, as was argued above, a prerequisite for liberation of DNA from this association. In reality, accumulation of ss breaks apparently has to increase the number of sites of alkaline (or heat)-induced DNA unwinding, in order to facilitate DNA release from the nucleoskeleton 'stud'.

In the next experiments we studied the effects of DNA breakage induced by gamma irradiation or bleomycin treatment of HeLa cells on DNA elution patterns (the double-gradient procedure was used here). Control intact cells were evenly labeled with [14C]thymidine while the cells to be damaged were labeled with [3H]thymidine. The control and damaged cell samples were mixed, lysed and analysed together. It is clearly seen from Fig. 9A (left panel) that ss breaks, which are predominant upon

Fig. 8. NPC chromatography of DNA from HeLa nuclei digested with Bal31 (A) or S1 (B) nucleases. Exponentially growing HeLa cells were labelled with [3H]thymidine (0.5 μCi/ml) for 24 h. Isolated DNA-matrix complexes, either intact (○—○) or digested with a ss-specific nuclease (●—●) were subjected to the double-gradient procedure. The control samples were incubated under the same conditions as for those treated with a nuclease, except for omission of the enzyme.
Fig. 9. Effect of DNA damage on the chromatographic patterns. (A) Effect of gamma irradiation. One portion of continuously growing HeLa cells used as a control sample was labeled with \([^{14}\text{C}]\text{thymidine} (0.5 \mu\text{Ci} \text{ml}^{-1})\) for 24 h (○ ○), while the other portion, labeled for 24 h with \([^{3}\text{H}]\text{thymidine} (1.5 \mu\text{Ci} \text{ml}^{-1})\), was gamma-irradiated (200 Gy) (● ●). Both portions were mixed, lysed and used for preparing isolated nuclei, which were subjected to double-gradient NPC chromatography (left panel). Both the control (○ ○) and gamma-irradiated HeLa cells (● ●) were analyzed separately by alkaline sucrose density gradient centrifugation (right panel). (B) Effect of bleomycin treatment. One portion of continuously growing HeLa cells used as a control sample was labeled with \([^{14}\text{C}]\text{thymidine} (0.5 \mu\text{Ci} \text{ml}^{-1})\) for 24 h (○ ○), while the other portion, labeled for 24 h with \([^{3}\text{H}]\text{thymidine} (1.5 \mu\text{Ci} \text{ml}^{-1})\), was treated with bleomycin (40 \(\mu\text{g} \text{ml}^{-1}\) for 30 min (● ●). Both portions were mixed, lysed and used for preparing isolated nuclei, which were subjected to double-gradient NPC chromatography (left). Both the control and treated cells were analyzed separately by alkaline sucrose density gradient centrifugation (ordinate, % of the total label recovered in a sedimentation fraction) (right panel).

Discussion

The nucleoskeleton, its structure and function are subjects of active current discussion: opinions range from viewing its origin as an artefact (reviewed by Djondjurov et al. 1986; Cook, 1988) to considering it as a receptacle for multienzyme complexes executing the fundamental nuclear processes (reviewed by Bodnar, 1988; Cook, 1988). The complexity and lability of DNA–matrix interactions (Kaufman and Shaper, 1984), the imperfection and diversity of the methods used for matrix isolation, and the high probability of some artefactual interactions are reasons for the conflicting results. From this point of view, an analytical, rather than the usual preparatory, procedure of isolation of DNA fragments from various nuclear compartments may be of significant importance in unraveling a hierarchy of DNA–protein interactions along the chromatin fiber.

Utility of the method

We think that NPC chromatography makes it possible to avoid a number of artefacts inherent in the usual isolation procedures, such as the formation of disulfide bonds during prolonged isolation of nuclei (Kaufman and Shaper, 1984), protein aggregation and redistribution during the exposure of the nuclei to high ionic strength (Djondjurov et al. 1986), and the breakdown of transcriptional complexes at low ionic strength (Razin et al. 1986).

First, the demands of the authors who assign top priority to the integrity and intactness of nuclei used for analysis can be easily satisfied by the ability of the method to permit the use as starting material of freshly prepared...
nuclei or even whole cell lysates (Lichtenstein et al. 1982). Second, NPC chromatography seems to be less prone than some other methods to high salt-induced protein redistribution. In fact, the striking increase in ionic strength routinely practised for dehistonization of isolated nuclei can lead, apart from possible osmotic phenomena, to the liberation of large masses of proteins and unbound DNA with a high potential for artefactual interactions. This is probably not the case when the concentration of a dissociating agent rises gradually and relatively slowly as it does in NPC chromatography. Besides this, the adsorbent Celite 545 used for chromatography is well known to bind proteins efficiently and as such should prevent the dissociated proteins from secondary interactions with unbound nucleic acids.

Differential dissociation of chromatin digests is able to reveal, in principle, a whole range of DNA–protein interactions (and identify, at first approximation, their chemical nature) instead of the usual and relatively crude separations of DNA in nucleosome-depleted and detached fractions. And, finally, the finding that some DNA damage (ss and ds breaks, crosslinks) can be revealed by chromatographic shifts suggests the applicability of the method to this purpose as well.

**Two types of DNA–nucleoskeleton interaction and hierarchy of chromatin loops**

This hypothesis, put forward more than 15 years ago (Dingman, 1974), was later experimentally confirmed by Laemmli and coauthors (Lebkowski et al. 1982a,b), who presented evidence for two levels of DNA folding in histone-depleted HeLa interphase nuclei, implying the existence of type I and type II nuclear scaffolds. The demonstration of two different types of DNA sequences involved in attachment of DNA to the nuclear matrix (Yarovsky and Razin, 1983) made an additional contribution to this idea. And, finally, competition studies on scaffold attached regions (SAR)–scaffold interactions in vitro also revealed the heterogeneity of DNA-binding sites (Amati and Gasser, 1988; Tsutsui et al. 1988; Mielke et al. 1990). And, finally, an analysis of supragenic loop organization in *Drosophila* embryos enabled the authors to discern strong SARs that were presumably organizing the DNA molecule as consecutive loops and weak SARs subdividing the basic loops into smaller ones (Surdej et al. 1990).

These and other data are the basis of the model proposed by Bodnar (1988), which suggests that there are both stable contacts between chromatin fibers and the nuclear periphery (where ori regions, enhancers and consensus sequences of topoisomerase II are concentrated) and dynamic (labile) multiple contacts with the inner network, the latter mediating transcription. The data we obtained on two types of DNA–nucleoskeleton association (DNA-I and DNA-II) were consistent with the above-mentioned observations. And, finally, the finding that the analogous fractions were also revealed in isolated nuclei without previous treatment with 2 M NaCl led us to conclude that both DNA-I and DNA-II fractions can be observed in the cell in vivo. Nevertheless, the possibility exists that electrophoretic removal of detached fragments from encapsulated nuclei by Jackson et al. (1990) is more effective than the preparatory extraction of digested nuclei with 2 M NaCl by the method of Berezney and Buchholtz (1981). In other words, the possibility cannot be ruled out that some fraction of DNA-I and DNA-II (not shown) might be released from the nuclear matrix. It is quite possible that some specific DNA–protein complexes are in equilibrium between the free and the nucleoskeleton-attached state.

Preparations of DNA-I (1 and 2) and DNA-II(1) all manifest the presence of a nucleosomal ladder that is absent from preparations of nucleoskeleton-attached DNA obtained by Jackson et al. (1990). If present, nucleosome-size molecules indicate the presence of the ordinary (nucleosomal) organization of chromatin in the vicinity of DNA–nucleoskeleton attachment sites, namely the tight one, is the site of nascent DNA.

Revelation of a high heterogeneity of DNA–nucleoskeleton attachment sites makes it possible to estimate the sizes of corresponding loops. It was found that in HeLa cells the DNA-II(2) and DNA-II(1) subfractions of tightly bound DNA form loops of 300–390 kbp and 200–208 kbp long, while those of weakly bound DNA (DNA-I(2) and DNA-I(1)) form loops 110–150 kbp and 90–130 kbp long, respectively. Being superimposed, all these attachment sites would form loops of approximately 40 kbp long, i.e. about two times less than the estimates made by Jackson et al. (1990) for the same subject.

The contradiction noted can be explained in different ways. First, some artefactual binding of small DNA fragments to the nucleoskeleton probably takes place during the chromatographic procedure. Though this phenomenon cannot be ruled out completely, it seems unlikely in the light of the discussion above. Second, the method elaborated by Jackson et al. (1990) permits them to isolate exactly the nucleoskeleton-attached DNA fragments, maximally avoiding perturbations caused by non-physiological conditions. As for NPC chromatography, it probes for DNA–protein interactions as such, with no indication as to whether they belong to the nucleoskeleton or any other subnuclear structure. An attribution of DNA-I and DNA-II fractions as the nucleoskeleton-attached ones was performed using an independent method of subnuclear fractionation. It was found that free nucleosomes contain DNA-0 as the most predominant fraction, while DNA–matrix complexes obtained with 2 M NaCl contain almost exclusively DNA-I and DNA-II (not shown). The finding that the analogous fractions were also revealed in isolated nuclei without previous treatment with 2 M NaCl led us to conclude that the DNA-I and DNA-II attachment sites occur in vivo. Nevertheless, the possibility exists that electrophoretic removal of detached fragments from encapsulated nuclei by Jackson et al. (1990) is more effective than the preparatory extraction of digested nuclei with 2 M NaCl by the method of Berezney and Buchholtz (1981). In other words, the possibility cannot be ruled out that some (presumably relatively small) portion of DNA-I and DNA-II may belong to free chromatin fibers rather than to the nucleoskeleton itself. It is quite possible that some specific DNA–protein complexes are in equilibrium between the free and the nucleoskeleton-attached state.

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attachment sites, while, on the contrary, the absence of small molecules may indicate some kind of protein protection of DNA molecules at nearby attachments sites and/or their deficiency at recognition sites for the restriction enzyme used. The possible reasons for such a contradiction were discussed above. On the other hand, this raises a question concerning the size of the attached region itself. Unlike large size fragments whose attachments to the nucleoskeleton can appear rather long in length, DNA fragments as small as mono- or dinucleosomes are obviously attached to the nucleoskeleton via a region much smaller than that (0.7 kbp long) determined for HeLa cells by Jackson et al. (1990). This finding lends further support to the idea of high heterogeneity of sites where DNA and nucleoskeleton interact with one another.

The authors thank Dr P. S. Agutter for critical reading of the manuscript.

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