Agarose gel electrophoretic evidence for domains of nuclear DNA linked with bonds cleavable with sulfhydryl molecules

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Complexes of intact nuclear DNA with proteins undisssociable by 2.0 M NaCl and nonionic detergents were analyzed by agarose gel electrophoresis following physical or enzymatic fragmentation. Sulfhydryl molecules converted these DNAs (but not the bacteriophage λ DNA) into smaller-M_r forms. Following limited restriction endonulease digestion of complexes with *PstI* most of the nuclear DNA formed a high-molecular-mass band in the 60–110 kbp range. These 60–110 kbp fragments, releasable from the rest of nuclei by sulfhydryl molecules, have similar sizes to nuclear DNA loops detected by other techniques and may derive from supranucleosomal organizational units in the chromatin complex.

Nuclear DNA *Nuclear matrix* *Sulfhydryl protein* *Disulfide protein* *DNA domain* *Electrophoresis*

1. INTRODUCTION

Lysis of cells or nuclei with nonionic detergents and high concentrations of NaCl or polyanions causes dissociation of histones and most of the nonhistone proteins from nuclear DNA. The intact nuclear DNA that is left behind is associated with a protein framework variously called a cage [1] or scaffold [2]. Proteins of these residual structures include most or all of the proteins of nuclear lamina matrices obtained by extensive nucleolytic digestion of nuclei [3,4]. Elements of these nuclear protein skeletal structures appear to serve as sites for transcription [5], heterogenous nuclear RNA processing [6] and replication [7].

Demembranized nuclei as well as complexes of intact nuclear DNA with nuclear protein skeleton structures display slowed sedimentation through neutral sucrose gradients when treated with 2ME or similar sulfhydryl molecules [8–10], apparently due to decondensation or unfolding of DNA [8–10]. Indeed, 2ME-induced unfolding of nuclear DNA can be visualized by both light and electron microscopy [10–13]. Release of DNase I-nicked DNA (as chromatin particles) from the relatively DNase I-resistant nuclear fraction (nuclear protein matrix) by 2ME has also been found [14]. Other studies provided evidence that proteins undissociable from nuclear DNA with nonionic detergents and 2.0 M NaCl are rich in intermolecular S–S bonds [4]. Here we show directly by agarose gel electrophoresis that bonds breakable with sulfhydryl molecules link regions of nuclear DNA with one another and that at least a part of the molecules participating in these bonds are sensitive to digestion with proteinase K. In addition we present evidence that the nuclear DNA in mammalian cells may be organized into 60–110 kbp domains. These domains are freed from the rest of the nuclear protein skeleton when treated with sulfhydryl molecules.

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Abbreviations: kbp, kilo basepairs; 2ME, 2-mercaptoethanol

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2. MATERIALS AND METHODS

The complexes of positively supercoiled intact nuclear DNA with proteins undissociable from DNA by Nonidet P-40 (0.8%, v/v) and 2.0 M NaCl, called here nucleoids following the terminology of [1], were prepared using live cells as described [4] except that equimolar concentrations of iodoacetamide replaced iodoacetic acid in the solutions used. The pelleted nucleoids were washed several times with deionized water (pellets are firm and can be handled unbroken with the aid of a polypropylene pipette tip) and used either fresh or following 2–8 weeks storage on liquid nitrogen. Nucleoid pellets were washed twice with 50 mM $MgCl_2$ and electrophoresed through 1% agarose gels either intact or after mechanical shearing and/or restriction endonuclease digestion (submerged gel electrophoresis with Tris-acetate-EDTA buffer [15]). Equal aliquots from nucleoid samples were placed in Eppendorf tubes, to half of these aliquots 2ME or α-thioglycerol was added to 3% (v/v) final concentration and to the other half ethanol was added to 3% (v/v). The 2ME-treated and untreated samples were run under identical conditions in separate tanks. 'Equal aliquots' from intact nucleoid pellets were taken by cutting pellets in the middle with a scalpel. Mechanical shearing of nucleoid pellets was done in the *Pst*I digestion buffer (see below) by hits on the pellet with a polypropylene pipette tip and by repeated passage through the tip until a homogeneous solution was obtained. For digestion with restriction endonuclease *Pst*I (at 37°C), nucleoids were placed in a buffer consisting of 50 mM NaCl, 25 mM $MgCl_2$, 2 mM iodoacetamide, 100 μg/ml bovine serum albumin, 25 mM Tris-HCl, pH 7.8, and added with indicated amounts of *Pst*I from stocks obtained from Pharmacia (NJ, USA) or IBI (CT, USA). Additional digestions of nucleoids with DNase I, RNase A and proteinase K (Sigma, MO; highest grades) were done essentially as described [15].

3. RESULTS

Electrophoresis of intact nucleoid pellets resulted in little DNA entry into gels (fig.1R,T,V,X). 2ME treatment of intact nucleoids prior to electrophoresis caused increases in the entrance of DNA into gels but the majority of DNA still remained at the origins (fig.1S,U,W,Y). Shearing of nucleoids as described in section 2 enhanced movement of DNA into gels but once again the majority of DNA was held at the origins (fig.1B,F,J,N). DNA from these sheared nucleoids entered gels in increased quantities and moved greater distances when samples were exposed to 2ME (fig.1C,G,K,O).

When nucleoids were digested extensively with restriction endonuclease *Pst*I and subjected to electrophoresis, DNA migrated throughout the length of the gels (fig.1D,H,L,P). When such restriction digested nucleoids were treated with 2ME or with

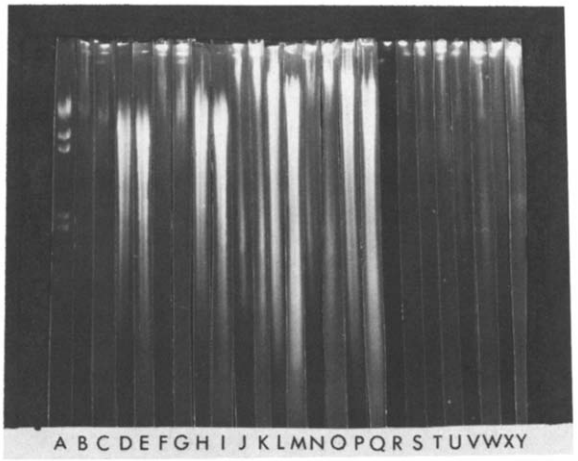

Fig.1. Agarose gel electrophoresis of intact, mechanically sheared and *Pst*I-digested nucleoids. (A) *Hind*III digest of bacteriophage λ DNA, (B–Y) nucleoids. Types and numbers of cells giving rise to nucleoids and treatments for nucleoids: (B) primary rat embryo fibroblasts (1.6×10^6), sheared; (D) same as B but *Pst*I digested; (F) Neo-1 cells (1.6×10^6), sheared; (H) same as F but *Pst*I digested; (J) F9 mouse teratocarcinoma cells (1.6×10^6), sheared; (L) same as J but *Pst*I digested; (N) F9 cells differentiated with retinoic acid (1.6×10^6), sheared; (P) same as N but *Pst*I digested; (R) same as B but intact; (T) Neo-1 cells (2.7×10^6), intact; (V) F9 cells (2.1×10^6), intact; (X) F9 cells differentiated with retinoic acid (3.5×10^6), intact. C, E, G, I, K, M, O, Q, S, U, W, Y represent identical samples to the preceding lanes except for 2ME treatment (i.e. C same as B, E same as D, etc.). *Pst*I digestions were done in 160 μl volumes with 270 units enzyme for 120 min ('extensive digestion'). Neo-1 and myc-1 cells (fig.2) are rat cells transfected with neomycin resistance gene and c-myc, respectively [18].

α-thioglycerol and then electrophoresed, a distinct shift to smaller DNA sizes was seen with all cell types studied (fig.1E,I,M,Q). Following this treatment most of the DNA seen otherwise at origins and at high-M_r regions moved further down and also additional low-molecular-mass DNA fragments (as low as 2 kbp or smaller) appeared (fig.1E,I,M,Q).

Nucleoids subjected to limited digestion with *Pst*I displayed larger DNA molecules than the extensively digested samples, as would be expected (figs 2 and 3). High-M_r regions of the gel lanes of limited digested–non-2ME-exposed nucleoids generally showed diffuse fluorescence (figs 2B,D,3A,E,I) but on occasion hints of a high-M_r band were present (e.g. fig.2B). Aliquots from the same samples that were treated with 2ME or α-thioglycerol, however, clearly displayed a high-molecular-mass band which is estimated from the *Hin*dIII digest of bacteriophage λ DNA run in the same gels to be within the 60–110 kbp size range (figs 2C,E,3B,F,J). This high-M_r banding, which was associated with a decrease or disappearance of ethidium bromide fluorescence from the gel region

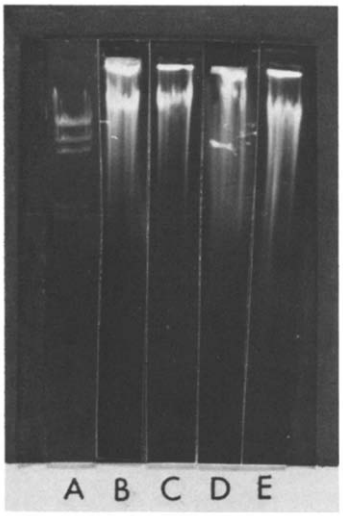

Fig.2. Agarose gel electrophoresis of nucleoids subjected to limited *Pst*I digestion. Nucleoids from 2.5×10^6 neo-1 cells (B) and 2.5×10^6 myc-1 cells (D) were digested with 28 units *Pst*I in a 100 μl volume for 30 min ('limited digestion') and electrophoresed. C is identical to B and E to D except for 2ME treatments of C and E. Lane A is *Hin*dIII digest of bacteriophage λ DNA treated with 2ME.

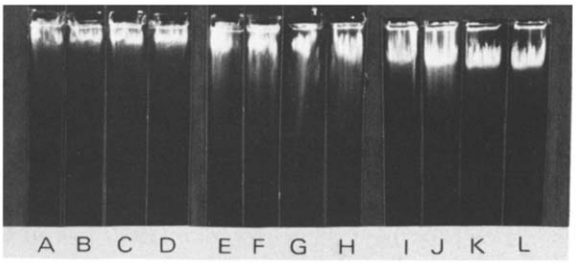

Fig.3. Influence of proteinase K on the high-M_r band formation by limited *Pst*I-digested nucleoids. Lanes: A–H, nucleoids from normal mouse spleen cells and I–L, from normal human mononuclear blood cells (3.6×10^6 per well). Nucleoids limited-digested with *Pst*I (A,E,I) and their 2ME-treated (B,F,J), proteinase K-digested (C,G,K), proteinase K-digested–2ME-treated (D,H,L) equivalents are shown. For proteinase K digestion, 5 μl of a 0.5 M EDTA, 2% (w/v) SDS solution was added to each 100 μl *Pst*I digest and after mixing, 5 μl of a 40 mg/ml proteinase K solution added. Samples with and without proteinase K were incubated at 37°C for 45 min further and electrophoresed in parallel. A–D were run in 7 cm gels. E–H and I–L were run in 16 cm gels and until bromophenol blue marker run out of the gels.

between the band and origin, was seen with all cell types regardless of the tissue or species of origin. Electrophoresis with longer gels or for longer periods (that allow the bromophenol blue marker to run out of the gels) gave better resolution of the high-M_r band (cf. fig.3B,F,J).

Additional digestion of the *Pst*I-digested nucleoids with proteinase K prior to electrophoresis was by iself sufficient for formation of the high-M_r band by non-2ME-exposed nucleoids (fig.3C,G,K). A minimal effect of 2ME was found in further enhancing band formation by proteinase K-digested nucleoids (fig.3D,H,L). We also performed additional digestions of *Pst*I-digested nucleoids with RNase A and DNase I. While no influence of RNase A was detected with regard to the fluorescence pattern and band formation, DNase I quickly destroyed the high-M_r band and caused the complete disappearance of fluorescence from gel lanes when samples were digested for prolonged periods (not shown).

4. DISCUSSION

Electrophoretic mobilities of DNA fragments in

agarose gels under identical conditions would in principle be dependent on size and conformation. Considering that possible conversion of supercoiled DNAs to relaxed forms by 2ME would decrease rather than increase electrophoretic mobilities, greater migration of DNA molecules in gels following exposure to 2ME must be due to 2ME-induced formation of smaller molecular sizes. This 2ME effect does not appear to be attributable to causation of nonspecific DNA damage under our conditions because under the same conditions 2ME did not alter bacteriophage λ DNA (figs 1A,2A; and not shown). DNA fragments as short as a few kilo basepairs were identifiable as being linked with 2ME-sensitive bonds to other fragments or nuclear skeletal structures when nucleoids extensively digested with *Pst*I were analyzed. However, Southern blots of these gels and hybridization with cloned genomic probes are required to decide which higher-M_r fragments are converted to which shorter fragments by 2ME and to identify possible functional correlates of location along DNA of 2ME-sensitive bonds.

On the other hand, limited *Pst*I digestion of nucleoids clearly yielded 60–110 kbp fragments, a large proportion of which existed as part of still bigger complexes held together with 2ME-sensitive bonds. These 60–110 kbp fragments are indicated to be made primarily of DNA from their formation after *Pst*I action, high representation in the loaded material, resistance to proteinase K and RNase A, and extreme sensitivity to DNase I. Considering that extensive *Pst*I digestion of nucleoid DNA causes smearing patterns (fig.1) similar to those seen with phenol-extracted [15] nuclear DNAs digested with less enzyme for shorter periods (unpublished), the uncut *Pst*I sites on 60–110 kbp fragments must have relative protection against enzyme action. Regardless of the mechanism for this protection (e.g. due to conformational constraints imposed by ethidium bromide intercalation and positive supercoiling?), it cannot go unnoticed that the 60–110 kbp size range is comparable or identical to the size range of DNA loops detected previously by electron microscopy and other techniques in histone-depleted chromosomes and nuclei [1,2,16] and to the sizes of the largest chromatin fragments or domains cut from intact nuclei by mild nuclease digestion [17]. It is of further interest that the 60–110 kbp fragments were detected with all cell types studied regardless of the species or tissue of origin (differences in the high-molecular-mass band mobility between different cell types have been noted but no generalizations are yet possible). Also, most of all nucleoid DNA may consist of 60–110 kbp fragments (fig.3K,L). Taken together, these considerations favour representation by the 60–110 kbp DNA fragments of supranucleosomal organizational units in the nucleus. Our findings that proteinase K alone was capable of releasing high-molecular-mass fragments or domains from the rest of the nuclear structures, and that 2ME showed minimal further influence upon proteinase K-digested nucleoids suggest strongly that the 2ME-sensitive bonds holding 60–110 kbp fragments together as larger complexes belong at least in part to DNA-associated proteins.

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